IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Michael E. Spurlock	
Appln. No.	:	09/928,522	
Filed	:	August 13, 2001	Group Art Unit: 1647
Title	:	Bovine Leptin Protein, Antisense and Antibody	Examiner: C. J. Saoud
Docket No.	:	LL31.12-0015	

DECLARATION UNDER 37 C.F.R. § 1.131

MS Fee Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

SENT VIA EXPRESS MAIL

Express Mail No.: EV 485710565 US

Sir:

This Declaration is submitted to establish completion of the invention of the above-identified patent application in the United States by a date prior to December 27, 1995, which is the effective date of the prior art publication TELLAM et al. (Genbank Acc. No. U43943, Bos taurus OBESE mRNA, 27 January 1996), hereinafter referred to as the "Tellam submission." The Examiner cited the Tellam Submission in an Office Action mailed in the above-identified application on June 16, 2004. To establish the date of completion of the invention of this application, evidence that the invention was completed (reduced to practice) on or before December 26, 1995, which is a date earlier than the effective date (December 27, 1995) of the Tellam submission, is provided. Since the invention was reduced to practice prior to the effective date of the Tellam submission, there is no requirement to establish due diligence with regard to reduction to practice of the invention.

I, Michael E. Spurlock declare:

1. I am the sole inventor of the invention of the above-identified application, as defined in claims 22, 24-25, and 27-30 of the above-identified patent application.

2. Claim 22 of the above-identified application reads as follows:

22. (Currently Amended) An isolated single or double-stranded DNA molecule which encodes a bovine adipocyte polypeptide leptin, the DNA molecule consisting of the nucleotide sequence of SEQ ID NO:3 or a functional derivative thereof, wherein the DNA molecule or the functional derivative thereof hybridizes to the nucleotide sequence of SEQ ID NO:3 when placed in contact with the nucleotide sequence of SEQ ID NO:3 under stringent hybridizing conditions.

3. Claim 24 of the above-identified application reads as follows:

24. (Currently Amended) An isolated single or double-stranded DNA molecule which encodes a bovine adipocyte polypeptide leptin, the DNA molecule consisting of a nucleotide sequence of SEQ ID NO:3 or a variant thereof, wherein the DNA molecule or the variant thereof hybridizes to substantially all of the nucleotide sequence of SEQ ID NO:3 when placed in contact with the nucleotide sequence of SEQ ID NO:3 under stringent hybridizing conditions.

4. Claim 25 of the above-identified application reads as follows:

25. (Currently Amended) An isolated mRNA molecule which encodes a bovine adipocyte polypeptide leptin, the mRNA molecule encoded by a nucleotide sequence of SEQ ID NO:3 or a variant thereof, wherein the mRNA molecule or the variant of the mRNA molecule hybridizes to the mRNA molecule encoded by the nucleotide sequence of SEQ ID NO:3 when placed in contact with the mRNA molecule encoded by the nucleotide sequence of SEQ ID NO:3 under stringent hybridizing conditions.

5. Claim 27 of the above-identified application reads as follows:

27. (Currently Amended) An isolated mRNA molecule which encodes a bovine adipocyte polypeptide leptin, the mRNA molecule encoded by a nucleotide sequence SEQ ID NO:3 or a functional derivative thereof, wherein the functional derivative of the isolated mRNA molecule hybridizes to substantially all of the mRNA molecule

encoded by the nucleotide sequence of SEQ ID NO:3 when placed in contact with the mRNA molecule encoded by the nucleotide sequence of SEQ ID NO:3 under stringent hybridizing conditions.

6. Claim 28 of the above-identified application reads as follows:

28. (Currently Amended) An isolated mRNA molecule which encodes a bovine adipocyte polypeptide leptin, the mRNA molecule encoded by a nucleotide sequence SEQ ID NO:3 or a functional variant thereof, wherein the functional variant hybridizes to substantially all of the mRNA molecule encoded by the nucleotide sequence of SEQ ID NO:3 when placed in contact with the mRNA molecule encoded by the nucleotide sequence of SEQ ID NO:3 under stringent hybridizing conditions.

7. Claim 29 of the above-identified application reads as follows:

29. (Currently Amended) An isolated mRNA molecule which encodes a bovine adipocyte polypeptide leptin, wherein the isolated mRNA molecule hybridizes to substantially all of an mRNA molecule encoded by a nucleotide sequence of SEQ ID NO:3 when placed in contact with the mRNA molecule encoded by the nucleotide sequence of SEQ ID NO:3 under stringent hybridizing conditions.

8. Claim 30 of the above-identified application reads as follows:

30. (Currently Amended) An isolated mRNA molecule which encodes a bovine adipocyte polypeptide leptin, wherein the isolated mRNA molecule hybridizes to an mRNA molecule encoded by a nucleotide sequence of SEQ ID NO:3 when placed in contact with the mRNA molecule encoded by the nucleotide sequence of SEQ ID NO:3 under stringent hybridizing conditions.

9. On or before December 26, 1995, I completed (reduced to practice) the invention of the above-identified application, as defined in claims 22, 24-25, and 27-30 of the above-identified patent application, as evidenced by the following:

A. Copies of two documents are attached as Exhibits A-B of this Declaration. Each of the documents of Exhibits A-B were created on or before December 26, 1995. Various dates have been redacted from one or more of the documents of Exhibits A-

B. Each of the dates redacted from any of the documents of Exhibits A-B is on or before December 26, 1995.

B. During the period ending on or before December 26, 1995 when I completed (reduced to practice) the invention of the above-identified application, as defined in claims 22, 24-25, and 27-30 of the above-identified patent application, I was employed as a research scientist by Purina Mills, Inc.

C. During the period ending on or before December 26, 1995 when I completed (reduced to practice) the invention of the above-identified application, Dr. Shaoquan Ji, a post-doctorate researcher, worked under my direction in my laboratory at Purina Mills, Inc.

D. During the period ending on or before December 26, 1995 when I completed (reduced to practice) the invention of the above-identified application, Dr. Ji, pursuant to my instructions and under my direction, isolated at least two independent clones encoding for the bovine leptin polypeptide, or functional derivative(s), functional variant(s), or variant(s) of the bovine leptin polypeptide, presently defined in claims 22, 24-25, and 27-30 of the above-identified application, as explained more fully below:

i. First, Dr. Ji, pursuant to my instructions and under my direction, extracted total RNA from bovine adipose tissue that encoded for a bovine adipocyte polypeptide.

ii. The total RNA extraction referenced in ¶9.D.i. above was accomplished using an acidic guanidinium thiocyanate-phenol-chloroform extraction technique based on the method of Chomczynski and Saachi (Chomczynski and Sacchi, 1987, Analytic Biochemistry 162:156).

iii. After extracting the total RNA, Dr. Ji, pursuant to my instructions and under my direction, purified the total RNA and reverse transcribed the purified total RNA into a single-stranded bovine leptin cDNA product using a reverse transcriptase from Gibco BRL of Gaithersburg, MD.

iv. Then, Dr. Ji, pursuant to my instructions and under my direction, amplified the single-stranded bovine leptin cDNA product referenced in ¶9.D.iii. above to a double-stranded bovine leptin cDNA product via a Polymerase Chain Reaction (PCR) using synthetic DNA primers based on the published mouse leptin cDNA sequence.

v. The synthetic DNA primers referenced in ¶9.D.iv. above were designed to amplify the coding region of the bovine leptin gene while excluding the secretory signal at the 5'-terminal of the coding region.

vi. After amplifying the single-stranded bovine leptin cDNA product to a double-stranded bovine leptin cDNA product as described in ¶9.D.iv. above, Dr. Ji, pursuant to my instructions and under my direction, purified the double-stranded bovine leptin cDNA product and thereafter isolated the 450 base bovine leptin cDNA clone that consisted of the nucleotide sequence of SEQ ID NO:3, or a functional derivative, variant, or functional variant thereof, presently defined in claims 22, 24-25, and 27-30 of the above-identified application.

vii. After purifying the recombinant plasmid containing the 450 base clone, Dr. Ji, pursuant to my instructions and under my direction, repeated the process described in ¶¶9.D.i.-vi. to obtain several other additional independent bovine leptin cDNA clones.

E. During the period ending on or before December 26, 1995 when I completed (reduced to practice) the invention of the above-identified application, Dr. Ji, pursuant to my instructions and under my direction, sent at least two independent clones containing bovine leptin cDNA isolated pursuant to ¶¶9.D.i.-vii. to National Biosciences, Inc. (a commercial laboratory skilled in gene sequencing and gene sequencing protocol) with instructions to sequence the independent clones.

F. The sequence of one of the clones isolated pursuant to ¶¶9.D.i.-vii. that contained bovine leptin cDNA was disclosed in U.S. Patent Application No. 08/688,908 (now U.S. Patent No. 6,297,027) as the nucleotide sequence identified as SEQ ID NO:3. The above-identified application is a continuation-in part application of, and claims priority from, U.S. Patent Application No. 08/688,908.

G. The sequence of a second clone (referenced in ¶9.E.) isolated pursuant to ¶¶9.D.i.-vii. that contained bovine leptin cDNA was confirmed when Brian Hoffman of National Biosciences, Inc. sent Dr. Ji a letter (attached as Exhibit A of this Declaration) in response to Dr. Ji's instructions of ¶9.E. with an enclosed paper (attached as Exhibit B of this Declaration).

H. The second clone (referenced in ¶9.E. and ¶9.G.) included the sequence of a 450 base bovine leptin cDNA clone that I characterize as a functional derivative, functional variant, or variant of bovine leptin DNA encoding the nucleotide sequence of SEQ ID NO:3, as more fully explained below:

i. I first note the DNA sequence listing of Exhibit B is listed in reverse order. As a result, the first 20 nucleotides of the 450 base clone are the nucleotides identified as 450 to 430 (bottom row of Exhibit B).

ii. Therefore, the first twenty nucleotides of the DNA sequence provided in Exhibit B for the second clone are: AGGCCGTGCC TATCCAAAA.

iii. On the other hand, the first twenty nucleotides of SEQ ID NO:3 of the above-identified application are: AGGCCGTGCC TATCCAGAAA.

iv. Nucleotide 17 of SEQ ID NO:3 is a G or a guanine nucleotide.

v. On the other hand, nucleotide 17 of the 450 base clone of Exhibit B is an A or an adenine nucleotide.

vi. Since the sequence for the 450 base clone of Exhibit B includes an adenine DNA base (A) rather than the guanine DNA base (G) of SEQ ID NO:3, the 450 base clone of Exhibit B may be characterized as a functional variant, functional derivative, and/or variant of bovine leptin DNA encoding the nucleotide sequence of SEQ ID NO:3.

vii. Similarly, nucleotides 301 to 310 of the 450 base clone of Exhibit B are as follows: CGCAGTTCAG.

viii. Nucleotides 301 to 310 of SEQ ID NO:3 of the above-identified application are: CGCAGGTCAG

ix. Nucleotide 306 of the 450 base clone of Exhibit B is a T or a thymine nucleotide.

x. On the other hand, nucleotide 306 of SEQ ID NO:3 of the above-identified application is a G or a guanine nucleotide.

xi. Since the sequence for the 450 base clone of Exhibit B further includes a thymine DNA base (T) rather than the guanine DNA base (G), further support exists for my characterization of the 450 base clone of Exhibit B as a functional variant, functional derivative, and/or variant of bovine leptin DNA encoding the nucleotide sequence of SEQ ID NO:3.

I. I also note Exhibit B containing the 450 base clone encoding the nucleotide sequence of SEQ ID NO:3 DNA was submitted under 37 C.F.R. §1.131 during the prosecution of parent U.S. Patent Application No. 08/688,908 (now U.S. Patent No. 6,297,027).

J. Claim 22 defines an isolated single or double-stranded DNA molecule which encodes a bovine adipocyte polypeptide leptin, where the DNA molecule may consist of a functional derivative of the nucleotide sequence SEQ ID NO:3 that hybridizes to the nucleotide sequence of SEQ ID NO:3 when placed in contact with the nucleotide sequence of SEQ ID NO:3 under stringent hybridizing conditions. See ¶2.

K. The facts and evidence presented in ¶9.E, ¶9.G, and ¶9.H. above demonstrate that, during the period ending on or before December 26, 1995, I completed (reduced to practice) the invention of the above-identified application, as defined in claim 22 that is referenced in ¶2 and in ¶9.J above. The isolated bovine leptin cDNA clone that is the subject of ¶9.G and ¶9.H. is the functional derivative of the nucleotide sequence SEQ ID NO:3 defined in claim 22.

L. Claim 24 defines an isolated single or double-stranded DNA molecule which encodes a bovine adipocyte polypeptide leptin, where the DNA molecule may consist of a variant of the nucleotide sequence SEQ ID NO:3 that hybridizes to the nucleotide

sequence of SEQ ID NO:3 when placed in contact with the nucleotide sequence of SEQ ID NO:3 under stringent hybridizing conditions. See ¶3.

M. The facts and evidence presented in ¶9.E, ¶9.G, and ¶9.H. above demonstrate that, during the period ending on or before December 26, 1995, I completed (reduced to practice) the invention of the above-identified application, as defined in claim 24 that is referenced in ¶3 and in ¶9.L above. The isolated bovine leptin cDNA clone that is the subject of ¶9.G and ¶9.H. is the variant of the nucleotide sequence SEQ ID NO:3 defined in claim 24.

N. The facts and evidence presented in ¶9.E, ¶9.G, and ¶9.H. above are based on activities performed during the period ending on or before December 26, 1995 and therefore, coupled with the facts and evidence presented in ¶9.J. - ¶9.M. and ¶ 24 (see below), demonstrate completion, in the United States, of the invention of the above-identified patent application, as defined in claims 22 and 24, by a date prior to is the effective date (December 27, 1995) of the Tellam submission.

10. A cDNA (complementary DNA) molecule is a complementary copy ("clone") of an mRNA molecule; A cDNA molecule is prepared in a laboratory by reverse transcription of the complementary mRNA. See the definition of Complementary DNA (cDNA) at Jeremy Buhler, Glossary of Biotechnology Terms, 1998 (obtained from the Internet on 2-10-04 from http://www.cs.washington.edu/homes/jbuhler/research/array/glossary.html) (attached as Exhibit C of this Declaration under 37 C.F.R. § 1.131.

11. Consequently, based on the evidence presented in ¶10, those of ordinary skill in the art of microbiology would understand that when a cDNA molecule has been obtained and isolated,

this necessarily also means the mRNA molecule corresponding to the cDNA molecule has likewise been obtained and isolated.

12. Claim 25 defines an isolated mRNA molecule which encodes a bovine adipocyte polypeptide leptin, where the isolated mRNA molecule may be a variant of an mRNA molecule that is encoded by the nucleotide sequence SEQ ID NO:3 and hybridizes to the mRNA molecule encoded by the nucleotide sequence SEQ ID NO:3 when placed in contact with the mRNA molecule that is encoded by the nucleotide sequence SEQ ID NO:3 under stringent hybridizing conditions. See ¶4.

13. The facts and evidence presented in ¶9.E, ¶9.G, ¶9.H., ¶10, and ¶11 above demonstrate that, during the period ending on or before December 26, 1995, I completed (reduced to practice) the invention of the above-identified application, as defined in claim 25 that is referenced in ¶4 and in ¶12 above. The isolated bovine leptin cDNA clone that is the subject of ¶9.G and ¶9.H. is the complement of an mRNA molecule that in turn is the variant defined in claim 25 in terms of the mRNA molecule encoded by the nucleotide sequence SEQ ID NO:3.

14. Claim 27 defines an isolated mRNA molecule which encodes a bovine adipocyte polypeptide leptin, where the isolated mRNA molecule may be a functional derivative of an mRNA molecule that is encoded by the nucleotide sequence SEQ ID NO:3 and hybridizes to the mRNA molecule encoded by the nucleotide sequence SEQ ID NO:3 when placed in contact with the mRNA molecule that is encoded by the nucleotide sequence SEQ ID NO:3 under stringent hybridizing conditions. See ¶5.

15. The facts and evidence presented in ¶9.E, ¶9.G, ¶9.H., ¶10, and ¶11 above demonstrate that, during the period ending on or before December 26, 1995, I completed (reduced to practice) the invention of the above-identified application, as defined in claim 27 that is referenced in ¶5 and

in ¶14 above. The isolated bovine leptin cDNA clone that is the subject of ¶9.G and ¶9.H. is the complement of an mRNA molecule that in turn is the functional derivative defined in claim 27 in terms of the mRNA molecule encoded by the nucleotide sequence SEQ ID NO:3.

16. Claim 28 defines an isolated mRNA molecule which encodes a bovine adipocyte polypeptide leptin, where the isolated mRNA molecule may be a functional variant of an mRNA molecule that is encoded by the nucleotide sequence SEQ ID NO:3 and hybridizes to the mRNA molecule encoded by the nucleotide sequence SEQ ID NO:3 when placed in contact with the mRNA molecule that is encoded by the nucleotide sequence SEQ ID NO:3 under stringent hybridizing conditions. See ¶6.

17. The facts and evidence presented in ¶9.E, ¶9.G, ¶9.H., ¶10, and ¶11 above demonstrate that, during the period ending on or before December 26, 1995, I completed (reduced to practice) the invention of the above-identified application, as defined in claim 28 that is referenced in ¶6 and in ¶16 above. The isolated bovine leptin cDNA clone that is the subject of ¶9.G and ¶9.H. is the complement of an mRNA molecule that in turn is the functional variant defined in claim 28 in terms of the mRNA molecule encoded by the nucleotide sequence SEQ ID NO:3.

18. Claim 29 defines an isolated mRNA molecule which encodes a bovine adipocyte polypeptide leptin, where the isolated mRNA molecule hybridizes to substantially all of an mRNA molecule encoded by the nucleotide sequence SEQ ID NO:3 when placed in contact with the mRNA molecule encoded by the nucleotide sequence SEQ ID NO:3 under stringent hybridizing conditions. See ¶7.

19. The facts and evidence presented in ¶9.E, ¶9.G, ¶9.H., ¶10, and ¶11 above demonstrate that, during the period ending on or before December 26, 1995, I completed (reduced to practice) the invention of the above-identified application, as defined in claim 29 that is referenced in

¶7 and in ¶18 above. The isolated bovine leptin cDNA clone that is the subject of ¶9.G and ¶9.H. is the complement of the isolated mRNA molecule that in turn is defined in claim 29 as substantially hybridizing to the mRNA molecule encoded by the nucleotide sequence SEQ ID NO:3 under stringent hybridizing conditions.

20. Claim 30 defines an isolated mRNA molecule which encodes a bovine adipocyte polypeptide leptin, where the isolated mRNA molecule hybridizes to an mRNA molecule encoded by the nucleotide sequence SEQ ID NO:3 when placed in contact with the mRNA molecule encoded by the nucleotide sequence SEQ ID NO:3 under stringent hybridizing conditions. See ¶8.

21. The facts and evidence presented in ¶9.E, ¶9.G, ¶9.H., ¶10, and ¶11 above demonstrate that, during the period ending on or before December 26, 1995, I completed (reduced to practice) the invention of the above-identified application, as defined in claim 30 that is referenced in ¶8 and in ¶20 above. The isolated bovine leptin cDNA clone that is the subject of ¶9.G and ¶9.H. is the complement of the isolated mRNA molecule that in turn is defined in claim 30 as hybridizing to the mRNA molecule encoded by the nucleotide sequence SEQ ID NO:3 under stringent hybridizing conditions.

22. The facts and evidence presented in ¶9.E, ¶9.G, and ¶9.H. above are based on activities performed during the period ending on or before December 26, 1995 and therefore, coupled with the facts and evidence presented in ¶9.J. - ¶9.M., ¶ 10- ¶ 21, and ¶ 24 (see below), demonstrate completion, in the United States, of the invention of the above-identified patent application, as defined in claims 25 and 27-30, by a date prior to is the effective date (December 27, 1995) of the Tellam submission.

23. The facts and evidence presented in ¶9.E, ¶9.G, ¶9.H., ¶9.J. - ¶9.M., and ¶ 10- ¶ 22 above demonstrate that, during the period ending on or before December 26, 1995, I completed

(reduced to practice) the invention, as defined in claims 22, 24-25, and 27-30 of the above-identified application.

24. All of the acts referenced above, or documented by Exhibits A-B, occurred in the United States.

25. This Declaration is being submitted after issuance of a final Office Action in the above-identified application but is nonetheless timely-filed for at least two different reasons. First, as indicated in the Amendment After Final accompanying this Declaration, the Examiner improperly designated the present as Final. Second, this Declaration accompanies a first reply after final rejection for the purpose of overcoming a new requirement made in the final rejection, namely overcoming the Examiner's rejection of Applicant's prior timely filed Declaration Under 37 C.F.R. §1.131.

I declare that all statements made herein that are of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

INVENTOR:

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Lafayette, IN 47905
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Date: 12/16/04

Signature

GENERAL INFORMATION

a. Professional Appointments:

1) Postdoctoral Research Associate, University of Missouri, 1989-1990.
2) Primary Reviewer, U.S. Food & Drug Administration, Center for Veterinary Medicine, 1990-1991.
3) Postdoctoral Research Associate, Purdue University, 1991-1993.
4) Research Scientist, Purina Mills, Inc., 1993-1994.
5) Senior Research Scientist, Purina Mills, Inc., 1994-1996.
6) Research Manager, Purina Mills, Inc., 1997-1998.
7) Senior Research Manager, Purina Mills, Inc., 1998-1999.
8) Adjunct Assistant Professor, Purdue University, 1995-1999.
9) Assistant Professor, Purdue University, 1999-2002.
10) Associate Professor, Purdue University, 2002-present.

b. Awards & Honors:

1) National Pork Producers' Council, Innovative Basic Research Award, 1994.
2) National Pork Producers' Council, Innovative Basic Research Award, 1997.
3) ESCOP/ACOP Fellow, 2003-04.
4) School of Agriculture Research Award, 2004.

c. Memberships in Academic, Professional, and Scholarly Societies:

1) American Society of Animal Science
2) American Society for Nutritional Sciences
3) Endocrine Society
4) American Diabetes Association
5) Sigma Xi
6) Gamma Sigma Delta

A. EXCELLENCE IN RESEARCH

The overall goal of Dr. Spurlock's research is to develop innovative technologies that improve the efficiency of animal growth and support the consumer's desire for highly palatable, yet healthy, meat products. Experimental approaches encompass whole-animal and cell culture experimentation, and range from the collection of growth performance data to detailed evaluations of gene regulation and signal transduction. Two avenues of research are actively pursued. First, his laboratory seeks to define the roles that adipocyte-derived hormones and cytokines play in the regulation of the overall energy balance and partitioning among tissues. Secondly, he is actively involved in delineating the mechanisms by which stress and disease suppress growth and influence the composition of body weight gain. The latter project is currently directed at understanding immunological aspects of the adipocyte with a particular emphasis on the immunological role of adiponectin. Dr. Spurlock is also an active member of the Comparative Medicine Program, and is concentrating on developing the pig as a biomedical model. He has an ongoing collaborative project with Dr. Michael Sturek, Indiana University School of Medicine, to establish the Ossabaw breed of minipig as a model for the human metabolic syndrome. His research in this area focuses on inflammation in the adipocyte and its relationship to insulin sensitivity. Recent publications from his laboratory in this topic have received national and international media attention.

1. Published Work (*indicates primary author(s)):

a. Refereed papers:

1) Spurlock, M.E.*, and J.E. Savage*. 1992. Antioxidant activity of Japanese quail liver cytosol in the absence and presence of reduced glutathione. *Poultry Sci.* 71:928-931.

2) Spurlock, M.E.*, J.D. Browning*, and B.L. O'Dell*. 1992. Low zinc status in guinea pigs and chicks has no effect on reassembly rate of brain microtubules. *J. Nutr. Biochem.* 3:594-598.

3) Spurlock, M.E *, and J.E. Savage*. 1993. Effects of dietary protein, sulfur amino acids and antioxidants on fatty liver hemorrhagic syndrome induced in Japanese quail by dietary modifications. *Poultry Sci.* 72:2095-2105.

4) Spurlock, M.E.*, J.C. Cusumano, and S.E. Mills*. 1993. (-)-[^{3}H]-Dihydroalprenolol binding to β-adrenergic receptors in porcine adipose tissue and skeletal muscle membrane preparations. *J. Anim. Sci.* 71:1778-1785.

5) Spurlock, M.E.*, J.C. Cusumano, and S.E. Mills*. 1993. The affinity of ractopamine, clenbuterol and L-644,969 for the β-adrenergic receptor population in porcine adipose tissue and skeletal muscle membrane. *J. Anim. Sci.* 71:2061-2065.

6) Spurlock, M.E.*, J.C. Cusumano, S.Q. Ji, C.K. Smith, II, D.B. Anderson*, D.L. Hancock*, and S.E. Mills*. 1994. The effect of ractopamine on β-adrenoceptor density and affinity in porcine adipose and skeletal muscle tissue. *J. Anim. Sci.* 72:75-80.

7) McComb, M.A.* and M.E. Spurlock*. 1996. Expression of stress proteins in porcine tissues: Developmental changes and effect of immunological challenge. *J. Anim. Sci.* 75:195-201.

8) Spurlock, M.E.*, K.J. Hahn, and J.L. Miner*. 1996. Regulation of adipsin and body composition in the monosodium glutamate (MSG) mouse. *Physiol. Behav.* 60:1217-1221.

9) Spurlock, M.E.*, G.R. Frank, G.M. Willis, J.L. Kuske, and S.G. Cornelius. 1996. Effect of dietary energy source and immunological challenge on growth performance and immunological variables in the growing pig. *J. Anim. Sci.* 75:720-726.

10) Spurlock, M.E.*. 1997. Regulation of metabolism and growth during immune challenge: An overview of cytokine function. *J. Anim. Sci.* 75:1773-1783.

11) Bidwell, C.A.*, S.Q. Ji, G.R. Frank, S.G. Cornelius, G.M. Willis, and M.E. Spurlock*. 1997. Cloning and expression of the porcine obese gene. *Anim. Biotech.* 8:191-206.

12) Ji, S.Q.*, R.R. Scott, G.M. Willis, and M.E. Spurlock*. 1998. Partial cloning and expression of the bovine leptin gene. *Anim. Biotech.* 9:1-14.

13) Spurlock, M.E.*, G.R. Frank, S.G. Cornelius, S.Q. Ji, G.M. Willis, and C.A. Bidwell*. 1998. *Obese* gene expression in porcine adipose tissue is reduced by food deprivation but not by maintenance or submaintenance intake. *J. Nutr.* 128:677-682.

14) Spurlock, M.E.*, M.A. Ranalletta*, S.G. Cornelius, G.R. Frank, S.Q. Ji, G.M. Willis, A.L. Grant*, and C.A. Bidwell*. 1998. Leptin expression in porcine adipose tissue is not increased by endotoxin but is reduced by growth hormone. *J. Int. Cyto. Res.* 18: 1051-1058.

15) Ji, S.Q.*, G.R. Frank, S.G. Cornelius, G.M. Willis, and M.E. Spurlock*. 1998. Porcine somatotropin improves growth in finishing pigs without altering Calpain 3 (p94) or α-actin mRNA abundance and has a differential effect on calpastatin transcription products. *J. Anim. Sci.* 76:1389-1395.

16) Houseknecht, K.L *, C.A. Baile*, R.L. Matteri*, and M.E. Spurlock*. 1998. The biology of leptin: A review. *J. Anim. Sci.* 76:1405-1420.

17) Ji, S.Q.*, R.L. Losinski*, S.G. Cornelius, G.R. Frank, G.M. Willis, D.E. Gerrard*, F.F.S. Depreux*, and M.E. Spurlock*. 1998. Myostatin expression in porcine tissues: tissue specificity and developmental and postnatal regulation. *Am. J. Physiol.* 275: R1265-R1273.

18) Ji, S.Q.*, S. Neustrom*, G.M. Willis, and M.E. Spurlock*. 1998. Proinflammatory cytokines regulate myogenic cell proliferation and fusion but have no impact on myotube protein metabolism or stress protein expression. *J. Int. Cyto. Res.* 18:879-888.

19) Houseknecht, K.L.*, C.A. Bidwell*, C.P. Portocarrero, and M.E. Spurlock*. 1998. Expression and cDNA cloning of porcine peroxisome proliferator-activated receptor gamma (PPARγ). *Gene* 225:89-96.

20) Ji, S.Q.*, G.M. Willis, G.R. Frank, S.G. Cornelius, and M.E. Spurlock*. 1999. Soybean isoflavones genistein and genistin inhibit myoblast proliferation, fusion, and myotube protein synthesis. *J. Nutr.* 129:1291-1297.

21) McCracken, B.A.*, M.E. Spurlock*, M.A. Roos, F.A. Zuckermann, and H.R. Gaskins*. 1999. Weaning anorexia induced local inflammation in the piglet small intestine. *J. Nutr.* 129:613-619.

22) Spurlock, M.E.*, K.L. Houseknecht*, C.P. Portocarrero, S.G. Cornelius, G.M. Willis, and C.A. Bidwell*. 2000. Regulation of PPARγ but not *obese* gene expression by dietary fat supplementation. *J. Nutr. Biochem.* 11:260-266.

23) Houseknecht, K.L.*, C.P. Portocarrero, S.Q. Ji, R. Lemenager*, and M.E. Spurlock*. 2000. Growth hormone regulates leptin gene expression in bovine adipose tissue: correlation with adipose IGF-1 expression. *J. Endocrinol.* 164:51-57.

24) Leininger, M.T.*, C.P. Portocarrero, A.P. Schinckel, M.E. Spurlock*, C.A. Bidwell, J.N. Nielsen, and K.L. Houseknecht*. 2000. Physiological response to acute endotoxemia in swine: effect of genotype on energy metabolites and leptin. *Dom. Anim. Endocrinol.* 18:71-82.

25) Leininger, M.T.*, C.P. Portocarrero, C.A. Bidwell, M.E. Spurlock*, and K.L. Houseknecht*. 2000. Leptin expression is reduced with acute endotoxemia in the pig: correlation with glucose, insulin, and insulin-like growth factor-1 (IGF-1). *J. Int. Cyto. Res.* 20:99-106.

26) Spurlock, M.E.*, S.Q. Ji*, J.L. Kuske*, S.G. Cornelius, G.R. Frank, and G.M. Willis. 2001. Changes in the expression of uncoupling proteins and lipases in porcine adipose tissue and skeletal muscle during feed deprivation. *J. Nutr. Biochem.* 12:81-87.

27) Miner, J.L.*, K.J. Hahn, M.E. Spurlock*, and N.R. Staten*. 2001. Expression and complement D activity of porcine adipsin. *Protein Expr. Purif.* 23:14-21.

28) Spurlock, M.E.*, C.A. Bidwell*, K.L. Houseknecht*, J.L. Kuske*, C. Camacho-Rea*, G.R. Frank, and G.M. Willis. 2002. Nutritionally induced adipose hypertrophy in young pigs is transient and independent of changes in the expression of the *Obese* and peroxisome proliferator activated receptor genes. *J. Nutr. Biochem.* 13:112-120.

29) Mills S.E.*, M.E. Spurlock, and D.J. Smith. 2003. Beta-adrenergic receptor subtypes that mediate ractopamine stimulation of lipolysis. *J. Anim. Sci.* 81:662-668.

30) Houseknecht, K.L.* and M.E. Spurlock*. 2003. The regulation of lipid metabolism in peripheral tissues by leptin. *Nutr. Rev. Intl.* 16:83-96.

31) Ajuwon, K.M.*, J.L. Kuske*, D.M. Ragland, O. Adeola*, D.L. Hancock, D.B. Anderson, and M.E. Spurlock*. 2003. The regulation of IGF-1 by leptin in the pig is tissue specific and independent of changes in growth hormone. *J. Nutr. Biochem.* 14:522-530.

32) Ajuwon, K.M.*, J.L. Kuske*, D.B. Anderson, D.L. Hancock, K.L. Houseknecht, O. Adeola, and M.E. Spurlock*. 2003. Chronic leptin administration increases serum NEFA in the pig and differentially regulates PPAR expression in adipose tissue. *J. Nutr. Biochem.* 14:576-583.

33) Raman, P.*, S.S. Donkin*, and M.E. Spurlock*. 2003. Regulation of hepatic glucose metabolism by leptin in pig and rat primary hepatocyte cultures. *Am. J. Physiol.* 286: R206-216.

34) Ajuwon, K.M*, S.K. Jacobi*, J.L. Kuske, and M.E. Spurlock*. Interleukin-6 and interleukin-15 are selectively regulated by lipopolysaccharide in primary pig adipocytes. *Am. J. Physiol.* 286:R547-553.

35) Weber, T.E.* and M.E. Spurlock*. 2004. Leptin alters antibody isotype in the pig in vivo, and prevents dexamethasone-induced down-regulation of the anti-apoptotic gene, bcl-xL, in peripheral blood monocytes in vitro. *J. Anim. Sci.* 82:1630-1640.

36) Wulster-Radcliffe, M.C.*, J.A. Christian, J. Wang, and M.E. Spurlock*. 2004. The anti-inflammatory actions of adiponectin include the regulation of IL-6 and IL-10. *Biochem. Biophys. Res. Comm.* 316:924-929.

37) Jacobi, S.*, K.M. Ajuwon*, T.E. Weber*, J.L. Kuske, C.J. Dyer, and M.E. Spurlock*. 2004. Cloning and expression of porcine adiponectin, and its relationship to adiposity, lipogenesis, and the acute phase response. *J. Endo.* 182:133-144.

38) Ajuwon, K.M.* and M.E. Spurlock*. 2004. Direct regulation of lipolysis by interleukin-15 in primary pig adipocytes. *Am. J. Physiol.* 287:R608-611.

b. Manuscripts Submitted:

1) Wulster-Radcliffe, M.C.* and M.E. Spurlock*. Adiponectin suppresses proliferation and proinflammatory cytokine production in THP-1 monocytes, and induces caspase 3/7 activity. *J. Int. Cyto. Res.* ARP #17376.

2) Ajuwon, K.M.*, and M.E. Spurlock*. Adiponectin attenuates lipopolysaccharide-induced NFkB activation and IL-6 expression, and up regulates PPAR? expression in adipocytes. *Am. J. Physiol.*

c. **Research abstracts:**

1) Spurlock, M.E.* and J.E. Savage*. 1988. Modulation of fatty liver hemorrhagic syndrome in Japanese quail by glutathione and other antioxidants. *Poultry Sci.* 69 (Suppl.1):152.

2) Spurlock, M.E.* and J.E. Savage*. 1989. Enterocyte absorption of glutathione after oral administration and uptake by intestinal brush border vesicles. *Poultry Sci.* 68 (Suppl.1):139.

3) Spurlock, M.E.*, J.C. Cusumano, S.Q. Ji, D.B. Anderson, D.L. Hancock*, and S.E. Mills*. 1993. The effect of ractopamine on ß-adrenoceptor density and affinity in porcine adipose and skeletal muscle tissue. *J. Anim. Sci.* 71 (Suppl. 1):135.

4) Chavis, S.*, D.L. Hancock*, P.J. Ruwe-Kaiser, and M.E. Spurlock*. 1994. The effects of aspirin and *E. coli* lipopolysaccharide on growth, calpain and calpastatin activities in growing pigs. *J. Anim. Sci.* 72 (Suppl. 2):50.

5) Miner, J.L.*, K.J. Hahn, M.E. Spurlock*, P.J. Ruwe-Kaiser, and C.A. Baile. 1994. Porcine adipsin; cloning, expression, complement activity, and effect of fasting on serum concentration. *J. Anim. Sci.* 72 (Suppl. 1):74.

6) Gerrard, D.E.*, A.L. Grant*, C.S. Okamura, T.V. Moran, and M.E. Spurlock*. 1994. Developmental expression and location of IGF-1 and IGF-II mRNA in porcine skeletal muscle. *J. Anim. Sci.* 72 (Suppl. 1):160.

7) Mills, S. E.* and M.E. Spurlock*. 1995. Tissue and species variation in the activation of adenylate cyclase by β-adrenergic agonists. *J. Anim. Sci.* 73 (Suppl. 1):145.

8) McComb, M.A.*, P.J. Ruwe-Kaiser, and M.E. Spurlock*. 1995. Expression of stress proteins in developing pigs and in growing pigs challenged with endotoxin. *J. Anim. Sci.* 73 (Suppl. 1):64.

9) Ji, S.Q., S. Neustrom*, and M.E. Spurlock*. 1996. Effect of tumor necrosis factor alpha on protein metabolism in L8 myotube cultures. *J. Anim. Sci.* 74(Suppl. 1):150.

10) Spurlock, M.E.*, M.A. McComb*, and M.A. Roos. 1996. Effect of weaning and weaning diet on circulating acute phase proteins and intestinal stress protein expression in the pig. *J. Anim. Sci.* 74 (Suppl. 1):36.

11) Ji, S.Q.*, G.R. Frank*, and M.E. Spurlock*. 1996. Effect of amino acid deficiency and restricted energy intake on the expression of p94 and related genes in skeletal muscle of growing pigs. *FASEB J.* 10(3):A768.

12) Frank, G.R.*, M.E. Spurlock*, S.G. Cornelius, G.M. Willis, and M.A. McComb*. 1997. Effects of group size on performance, fat depth, and serum concentrations of insulin-like growth factor-1 in growing-finishing pigs. *J. Anim. Sci.* 75 (Suppl. 1):37.

13) Ji, S.Q.*, G.M. Willis, S.G. Cornelius, and M.E. Spurlock*. 1997. Soybean genistein inhibits myoblast proliferation, differentiation, and myotube protein synthesis. *J. Anim. Sci.* 75 (Suppl. 1):56.

14) McComb, M.A.*, J.W. Frank, A.P. Schinckel*, M.E. Spurlock*, B.T. Richert*, P.V. Malven, and A.L. Grant. 1997. Interactive effects of rearing environment, pig genotype, and antibiotic therapy on growth, serum IGF-1, and acute phase proteins. *J. Anim. Sci.* 75 (Suppl. 1):85.

15) Spurlock, M.E.*, M.A. McComb*, S.G. Cornelius, G.R. Frank, G.M. Willis, and A.L. Grant*. 1997. Effect of growth hormone and immune challenge on serum concentrations of insulin-like growth factor-1 and other components and adipose adipsin expression. *J. Anim. Sci.* 75 (Suppl. 1):85.

16) Holck, J.T.*, A.P. Schinckel*, J.L. Coleman, V.M. Wilt, E.L. Thacker, M.E. Spurlock, A.L. Grant*, P.V. Malven*, M.K. Senn, and B.J. Thacker. 1997. Environmental effects on the growth of finisher pigs. *J. Anim. Sci.* 75 (Suppl. 1):246.

17) Ji, S.Q.*, G.R. Frank, S.G. Cornelius, G.M. Willis, and M.E. Spurlock*. 1997. Effect of porcine somatotropin on skeletal muscle p94, calpastatin and α-actin gene expression in finishing pigs. *J. Anim. Sci.* 75 (Suppl. 1):163.

18) Ji, S.Q.*, G.M. Willis, R.R. Scott, and M.E. Spurlock*. 1997. Partial cloning of the bovine leptin gene and its expression in adipose depots and in cattle before and after finishing. *J. Anim. Sci.* 75 (Suppl. 1):167.

19) Spurlock, M.E.*, S.G. Cornelius, G.R. Frank*, and G.M. Willis. 1998. Growth performance and immunological variables in pigs fed different fat sources and subjected to multiple immunological challenges. *J. Anim. Sci.* 76 (Suppl. 2):59.

20) Ji, S.Q.*, R.L. Losinski*, S.G. Cornelius, G.R. Frank, G.M. Willis, and M.E. Spurlock*. 1998. Myostatin expression in porcine tissues: ontogeny, tissue specificity, and physiological regulation. *J. Anim. Sci.* 76 (Suppl. 2):42.

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22) Ji, S.Q.*, G.R. Frank, G.M. Willis, S.G. Cornelius, and M.E. Spurlock*. 1998. Myostatin expression in porcine longissimus muscle is not altered by dietary amino acid or energy intake restriction. *J. Anim. Sci.* 76 (Suppl. 2):40.

23) Spurlock, M.E.*, S.G. Cornelius, G.R. Frank, and G.M. Willis. 1998. Growth performance of finishing pigs fed diets with or without supplemental vitamins and trace minerals and subjected to multiple immunological challenges. *J. Anim. Sci.* 76 (Suppl. 2):53.

24) Ji, S.Q.*, G. Frank, S. Cornelius, G. Willis, and M.E. Spurlock*. 1998. Expression of skeletal muscle translation initiation factor eIF-4E is altered with age and fasting in young pigs. *FASEB J.* 12(4):A950.

25) Houseknecht, K.L.*, C.A. Bidwell*, C.P. Portocarrero, M.E. Spurlock*, and A. Heady. 1998. Cloning and regulation of porcine peroxisome proliferator-activated receptor γ (PPARγ). *J. Anim. Sci.* 76 (Suppl. 1):116.

26) Brandebourg, T.D.*, M.E. Spurlock*, and S.E. Mills*. 1998. The role of C/EBPα in mediating the antilipogenic effect of pST n finishing pigs. *J. Anim. Sci.* 76 (Suppl. 1):119.

27) Spurlock, M.E.* 1998. Physiological roles of leptin, myostatin, and other cytokines. *J. Anim. Sci.* 76 (Suppl. 1):120.

28) Portocarrero, C.P.*, K.L. Houseknecht*, S.Q. Ji, R.P. Lemenager, and M.E. Spurlock*. 1998. Somatotropin (bST) regulates leptin gene expression in growing cattle. *J. Anim. Sci.* 76 (Suppl. 1):128.

29) Leininger, M.T.*, C.P. Portocarrero, C.A. Bidwell, M.E. Spurlock*, J.N. Nielsen, and K.L. Houseknecht*. 1998. Response to immune challenge in pigs selected for high lean gain: Role of leptin. *J. Anim. Sci.* 76 (Suppl. 1):129.

30) Ji, S.Q.*, R.L. Godat*, G.M. Willis, G.R. Frank, S.G. Cornelius, and M.E. Spurlock*. 1999. Adipose and skeletal muscle expression of genes related to lipid metabolism in finishing pigs deprived of feed. *J. Anim. Sci.* 77 (Suppl. 2):19.

31) Spurlock, M.E.*, K.L. Houseknecht*, C.P. Portocarrero, S.G. Cornelius, and G.M. Willis. 1999. Regulation of PPAR but not leptin gene expression by dietary fatty acid supplementation. *J. Anim. Sci.* 77 (Suppl. 1):159.

32) Spurlock, M.E.*, J.L. Kuske*, C. Camacho-Rea*, G.R. Frank, G.M. Willis, and K.L. Houseknecht. 2001. Growth and body composition in growing-finishing pigs fed a liquid milk replacer diet throughout the nursery phase. *J. Anim. Sci.* 79(Suppl. 2):52.

33) N. Mathialagan* , C.J Dyer* , M.T Leininger* , K. Govinderajan, J.C Byatt , F.C Buonomo , and M.E. Spurlock*. 2002. Expression Profiling of low and high lean pigs from weaning to finish. Plant and Anim. Genome Conf. X, San Diego, CA. page 703. http://www.intl-pag.org/pag/10/abstracts/PAGX_703.html .

34) Raman, P.*, S.S. Donkin*, and M.E. Spurlock*. 2002. Comparative effect of leptin on hepatic gluconeogenesis in rat vs. porcine hepatocytes. Endocrine Soc. 84th Annual Meeting. San Francisco, California. (Abstract No. P3-409) page 587. http://www.intl-pag.org/pag/10/abstracts/PAGX_587.html

35) Ajuwon, K.M.*, J. Kuske*, D.L. Hancock, D.B. Anderson, and M.E. Spurlock*. 2002. Leptin reduces feed intake and increases serum fatty acid concentrations in growing pigs, but does not regulate acetyl Co-A carboxylase activity or PPARα expression in adipose tissue. *J. Anim. Sci.* 80 (Suppl. 1):154.

36) Bidwell, C.A.*, M.E. Spurlock*, and D.E. Moody*. 2003. Serial analysis of gene expression of pig adipose and muscle tissue. Plant and Anim. Genome Conf. XI, San Diego, CA. http://www.intl-pag.org/11/abstracts/P01_P69_XI.html .

37) Weber, T.E.*, and M.E. Spurlock*. 2003. The effect of exogenous leptin on immunological parameters in growing pigs. *J. Anim. Sci.* 81(Suppl. 1):200.

38) Schinckel, A.P.*, M.E. Spurlock*, B.T. Richert*, and T E. Weber*. 2003. Modeling health status-environmental effects on swine growth. 54th Annual Meeting of the European Assoc. for Anim. Prod. Book of Abstracts no 9. Rome, Italy, (Abstract number P1.3) page 355.

39) Wulster-Radcliffe*, M.C., J.A. Christian, J. Wang, and M E. Spurlock*. 2004. Adiponectin attenuates the induction of proinflammatory cytokines in pig peripheral blood monocytes by LPS, and increases the expression of IL-10. Endocrine Soc. 86th Annual Meeting. New Orleans, LA. (Abstract No. P3-524) page 594.

40) Wulster-Radcliffe*, M.C., and M E. Spurlock*. 2004. Adiponectin Attenuates the induction of proinflammatory cytokines in THP-1 monocytes by lipopolysaccharide, reduces proliferation, and increases caspase 3/7 activity. Endocrine Soc. 86th Annual Meeting. New Orleans, LA. (Abstract No. P3-523) pp. 593-594.

41) Barrett, B.L.*, K.M. Ajuwon*, J.L. Kuske*, and M.E. Spurlock*. 2004. Leptin enhances insulin-stimulated lipogenesis, but does not alter ADP-ribosylation or abundance of the inhibitory G-protein in pig adipocytes. Endocrine Soc. 86th Annual Meeting. New Orleans, LA. (Abstract No. P2-42) pp. 316-317.

42) Ajuwon, K.M.* and M.E. Spurlock*. 2004. Adiponectin attenuates the induction of IL-6 by lipopolysaccharide in primary pig adipocytes, and induces the ixpression of PPARγ2. Endocrine Soc. 86th Annual Meeting. New Orleans, LA. (Abstract No. P1-64) pp. 169-170.

43) Dyson, M.C.*, M. Wulster-Radcliffe*, M. Spurlock*, M. Alloosh, E.A. Mokelke, and M. Sturek*. 2004. Association of interleukin-6 with glucose intolerance and insulin resistance in a swine model of the metabolic syndrome. Endocrine Soc. 86th Annual Meeting. New Orleans, LA. (Abstract No. P2-38) page 316.

d. Papers published in conference proceedings:

1) Spurlock, M.E.*, and J.E. Savage*. 1993. Determination of true metabolizable energy content of bobwhite foods. Pages 109-114 in K.E. Church and T.V. Daily eds. Quail III: national quail symposium. Kansas Wildl. And Parks, Pratt.

2) Spurlock, M.E.*, M.A. McComb*, and M.A. Roos*. 1996. Effect of weaning and weaning diet on circulating acute phase proteins and intestinal stress protein expression in the pig. Proc. of the 14th Int. Pig Vet. Soc. Cong. Bologna, Italy. pp. 731-737.

3) Spurlock, M.E.*, G.R. Frank, S.G. Cornelius, R.P. Chapple, and G.M. Willis. 1996. Impact of environmental factors and disease on animal performance. Proc. of the Minn. Nutr. Conf., Minneapolis. pp. 211-226.

4) Spurlock, M.E.*, Weber, T.E.*, and Ajuwon, K.M.*. 2003. New Discoveries in adipocyte and muscle biology: Implications for endocrine and immune regulation of growth in pigs. Proc. Australian Pig Vet. Soc., Cairns, AU. pp. 51-57.

5) Spurlock, M.E.*, Jacobi, S.K.*, and Ajuwon, K.M.* 2004. The role of the adipocyte in energy regulation. Proc. Midwest Swine Nutr. Conf. pp. 51-57.

e. Invited Research Presentations:

I. State

1) Leptin: The long and short of a complex story. 2000. Purdue Univ. Interdepartmental Nutrition Cluster. Graduate student recruitment program, keynote address, West Lafayette, IN.

2) Leptin, adiponectin, and Toll-like receptors link the adipocyte to the immune response. 2001. Dept. of Vet. Pathobiol., Purdue Univ., West Lafayette, IN.

3) Cytokines and Toll-like receptors: The integration of growth and immunology. 2003. Dept. of Basic Medical Sciences, School of Vet. Med. (Dept. seminar), West Lafayette, IN.

4) Is there an immunological role for the adipocyte and adiponectin? 2003. Div.of Endocrinol. and Metab. Indiana Univ. School of Med., Indianapolis, IN.

5) Adipocytes, inflammation and the metabolic syndrome: Is there a link? 2004. Comparative Medicine Retreat, Purdue Univ., West Lafayette, IN.

6) Fat cells, pigs and people: A new look at an old enemy. 2004. School of Agriculture Research Award presentation and seminar, Purdue Univ., West Lafayette, IN.

7) The role of the adipocyte in energy regulation. 2004. Midwest Swine Nutr. Conf., Purdue Univ., West Lafayette, IN.

II. National

1) Regulation of skeletal muscle and adipose tissue growth and development by cytokines: An overview. 1995. Midwest Meeting, Amer. Soc. of Anim. Sci., Des Moines, IA, Stress and Anim. Growth Symp.

2) Leptin: A role in food animal production? 1997. Univ. of Nebraska, Lincoln.

3) The cytokinology of leptin. 1997. Amer. Soc. of Anim. Sci. Leptin Symp., Nashville, TN.

4) Impact of environmental factors and disease on animal performance. 1997. Minn. Nutr. Conf., Minneapolis, MN.

5) The impact of myostatin and selected cytokines on growth. 1998. Amer. Soc. of Anim. Sci. Animal Growth Symp., Denver, CO.

6) Leptin and Myostatin: The biology and possible food animal applications. 1998. Univ. of Georgia, Athens, GA.

7) Leptin and Myostatin: Targets for embryological manipulation of growth in avian species. 1998. Embrex, Inc., Raleigh, NC.

8) The status of leptin and myostatin in food animal biology. 1999. Pfizer Animal Health, Groton, CN.

9) Health challenges in food animals: Mechanisms and possible targets for prevention and alleviation. 1999. Univ. of Arkansas, Fayette.

10) The impact of disease challenge on growth potential: Recent findings and implications for selected cytokines. 1999. Amer. Soc. of Anim. Sci. Symp., Indianapolis, IN.

11) Leptin, leptin resistance, and fat accretion in the pig: Is there a relationship? 2000. Monsanto Co., St. Louis, MO.

12) Emerging perspectives on nutrition, body composition and immune function. 2003. Midwest Meeting, Amer. Soc. of Anim. Sci., Des Moines, IA.

13) New discoveries in adipocyte and muscle biology: are they new paradigms for growth and nutrition in pigs? 2003. Prince Agri Products, 23rd Annual Feed Ingredient Conf., Rochester, MN.

14) Adipocytes and inflammation: The evidence and the implications. 2004. Interdepartmental Nutrition Cluster Symp., The Ohio State Univ., Columbus, OH.

III. International

1) Growth potential and disease: A cellular perspective on why sick pigs perform poorly. 1999. American Association of Swine Practitioners Annual Meeting, St. Louis, MO.

2) Pigs, nutrition, and technology: Where are things headed? 2000. National Pork Producers Council. World Pork Exp., Pork Academy, Indianapolis, IN.

3) Linking energy balance to immune function through leptin and adiponectin. 2002. British Society of Anim. Sci. Symp., York, England.

4) New Discoveries in adipocyte and muscle biology: Implications for endocrine and immune regulation of growth in pigs (Co-Keynote Address with Dr. John Black, CSIRO (retired). 2003. Annual Meeting, Australian Pig Veterinary Society. Cairns, Australia. Similar presentations were made at CSIRO research locations in Perth, Jeelong, Toowomba, and Bendigo, and to the Australian Pork Limited technical staff, Canberra.

5) Adiponectin, the AMP-activated protein kinase and inflammation in adipocytes. 2004. National Autonomous University of Mexico (UNAM) and the National Institute of Nutrition and Medical Research, Mexico City, Mexico.

f. Patents:

1) Porcine Leptin: Sequence and Applications to Pork Production (joint inventor with Dr. Bidwell); #6,277,592.

2) Bovine Leptin: Sequence and Applications to Cattle Industries; #6,297,027.

3) Porcine adiponectin (pending; File Date: July 2003): Sequence and applications to obesity and inflammation.

g. Other research publications:

1) Leininger, M.T., C.P. Portocarrero, C.A. Bidwell, M.E. Spurlock, J.N. Nielsen, and K.L. Houseknecht. 1998. Effect of immune challenge on different genotypes: How sick do they get? Purdue University, Swine Day Report, pp. 1-8.

2) Spurlock, M. E., T.E. Weber, and K. Ajuwon. 2003. New discoveries in adipocyte and muscle biology: are they new paradigms for growth and nutrition in pigs? Proceedings of the 23rd Annual Prince Agri Products Feed Ingredients Conf. pp. 4-11.

2. Evidence of Creative Excellence:

Dr. Spurlock's research program can best be described as growth biology with an emphasis on nutrient intake and partitioning, as well as on the metabolic responses of animals to immunological and stress challenges. Additionally, Dr. Spurlock is an active participant in the Comparative Medicine Program, and is working with Dr. Merv Yoder, Indiana University School of Medicine, to establish a myocyte-adipocyte co-culture system using muscle-derived pluripotent cells. New knowledge in these areas is expected to facilitate the development of nutritional, management, and therapeutic strategies that will enhance the efficiency of animal production, and provide novel means of exploring communication pathways between adipocyte and myofibers that will ultimately benefit human health and animal production. He is also collaborating with Dr. Michael Sturek, Indiana University School of Medicine, to develop the Ossabaw pig as a model for the metabolic syndrome in humans. This pig is genetically predisposed to the metabolic syndrome, and progresses to the insulin-resistant pre-diabetic state when allowed ad libitum feed intake. As an additional component of this work, Dr. Spurlock is working with Drs. Moody and Gerrard to introgress the RN gene from Hampshire pigs onto the Ossabaw genetic background. The RN gene is a mutated AMP-activated kinase, and may enhance the utility of the Ossabaw pig as a model for the metabolic syndrome. The following sections describe successful endeavors and specific accomplishments, some of which occurred during Dr. Spurlock's adjunct faculty appointment.

a. The adipocyte:

Recent discoveries have established a new paradigm for the adipocyte, one in which the biological role of this cell extends far beyond the passive storage of excess energy. The discovery of leptin and its strong linkages to metabolic and immunological pathways have helped redefine the concept of the physiological role of adipocytes. These findings, coupled with the knowledge that adiponectin is only produced by the adipocyte and regulates phagocytosis and cytokine production by the macrophage, have provided a strong impetus to explore the linkages of the adipocyte to energy metabolism and immune function. Furthermore, the recent evidence that the adipocyte expresses the receptors for both gram-negative and gram-positive bacteria, and responds to direct stimulation, *in vitro,* underscores the need for knowledge regarding the immunological function of the adipocyte. Dr. Spurlock's research program addresses this new paradigm with emphasis on leptin and adiponectin. He has obtained evidence that leptin acts *directly* on cultured pig adipocytes to stimulate lipolysis, and is currently dissecting the signaling pathway that underlies this response. His work has also shown for the first time that lipopolysaccharide does indeed

stimulate *NFkB-mediated* gene expression and cytokine production in isolated adipocytes, and that adiponectin is a negative regulator of this response to lipopolysaccharide. Furthermore, his laboratory has identified an important differential response in adipocytes vs. macrophages in that cAMP *attenuates* NFkB signaling in macrophages and *stimulates* it in adipocytes. This finding has broad potential implications for cell-specific means of regulating IkBa, the cytosolic inhibitor of NFkB signaling. These two adipocyte-derived proteins may establish a strong integrated linkage among energy metabolism, adiposity, and immune function, and are particularly important to meat animal industries and human health because of the emphasis on reducing adiposity over the past two decades.

b. **Leptin:**

In collaboration with Dr. Bidwell, Dr. Spurlock's laboratory has focused on the metabolic regulation of leptin expression at the mRNA and protein levels, and the potential role of leptin during the inflammatory response. His group has provided much of the data available to date for the pig, and also developed the research tools required to study the role of leptin in cattle. This research provided some of the first indications of a regulatory relationship between growth hormone, IGF-1, and leptin expression, and also provided considerable evidence that nutritional state, independent of changes in fat mass, is a major determinant of leptin expression. This work has been expanded recently to show that exogenous leptin regulates IGF-1 expression in the liver and serum, independently of feed intake. Recent data from Dr. Spurlock's laboratory indicates that leptin also down regulates PPARγ1 and γ2 in adipose tissue, but has no effect on PPARa in adipose tissue, skeletal muscle, or liver. This points to a marked species difference in pigs (and likely humans) vs. rodent models. The regulation of PPARγ is perhaps casually related to a dedifferentiation event and suppression of lipogenic pathways. Dr. Spurlock has also identified important differences between leptin regulation in rodent models and pigs. Whereas leptin expression is markedly increased in rodents injected with lipopolysaccharide (to simulate bacterial infection), he has shown quite clearly that leptin expression in porcine adipose tissue is not responsive to lipopolysaccharide. This finding raises important questions regarding the potential roles of leptin in the immune response of the pig and the mechanisms by which they are accomplished. Recent evidence from Dr. Spurlock's group has shown for the first time in any species that leptin influences the IgG1:IgG2, and that leptin sustains anti-apoptotic gene expression in activated peripheral blood monocytes, in vitro, despite the presence of glucocorticoids. An understanding of the integrated actions of leptin in multiple metabolic states is a critical component of an effective overall strategy to improve growth and efficiency of food producing animals.

c. **Adiponectin:**

Dr. Spurlock's laboratory has also provided the first data that relate adiponectin production to the innate immune response, *in vivo* and *in vitro*. He is using isolated adipocytes, adipose explants, and 3T3-L1 cells to explore the response of the adipocyte to lipopolysaccharide and specific proinflammatory cytokines. Molecular probes have been constructed to study the expression of the porcine adiponectin gene, and Dr. Spurlock has obtained an antibody to pig adiponectin, and the recombinant protein for *in vitro* use. These research tools are being used to perform the first studies of the regulation of adiponectin

production by leptin, proinflammatory cytokines, and the regulation of adiponectin production in response to lipopolysaccharide, a model of gram-negative bacterial infection.

Recent experiments have resulted in significant findings. First of all, adiponectin blocks the induction of NF*k*B signaling in cultured adipocytes stimulated with bacterial endotoxin, but does not influence the response in stimulated myocytes. This points to differential signaling pathways across cell types, and is consistent with the recent discovery of two distinct receptors for adiponectin. Secondly, Dr. Spurlock's laboratory has determined that adiponectin inhibits lipogenesis in cultured pig adipocytes, and has linked this action of adiponectin to the AMP-activated protein kinase. Thus, there are clear immunological and endocrine actions of adiponectin, and these findings support the new paradigm for adipocytes as active immune and endocrine cells. Dr. Spurlock's group has now provided unequivocal evidence that adiponectin induces the expression of interleukin-10 (IL-10) in the macrophage. Because IL-10 is a major anti-inflammatory cytokine, this finding extends the actions of adipocyte-derived adiponectin to include not only suppression of proinflammatory cytokine production, but induction of anti-inflammatory cytokines. Thus, the adiponectin receptor and signaling pathway may provide a means of enhancing circulating IL-10 concentrations, which would be beneficial to animal production and human health.

Finally, Dr. Spurlock's laboratory has recently determined that interferon-? induces the expression of interleukin-15 in cultured adipocytes, and have now identified key elements of the signaling pathway responsible for this induction. They have now extended this finding to show for the first time in any species that this cytokine acts directly on the adipocyte to regulate lipolysis. This finding is novel, and provides further evidence that the adipocyte regulates nutrient flux because of the well documented regulation of protein accretion in muscle by this cytokine.

d. Growth-stress-cytokine axis:

During periods of immunological challenge and stress, immune and stress modulators orchestrate a reduction in food intake and alterations in peripheral tissue metabolism that diminish growth and repartition nutrients to support higher priority metabolic needs. Reductions in local and systemic concentrations of insulin-like growth factor 1 (IGF-1) may be a critical component of the mechanism by which growth is slowed and nutrients are repartitioned. Dr. Spurlock's laboratory has shown that exogenous leptin reduces IGF-1 expression in the liver of the pig, in parallel with a reduction in serum concentrations. Furthermore, this response is independent of feed intake. These data provide further evidence of a regulatory linkage between leptin and IGF-1. Dr. Spurlock has shown that the proinflammatory cytokines, interleukin-1 (IL-1) and tumor necrosis factor alpha (TNFα), suppress myoblast proliferation and differentiation, and that the effects of these immune modulators are additive. This finding illustrates the potential for disease exposure in the dam to impact the growth potential of her offspring. His group has also provided strong evidence that IGF-1 expression in skeletal muscle is reduced in parallel with serum IGF-1 during infection, independently of feed intake, and despite the administration of exogenous growth hormone. He has extended these findings to also show that serum IGF-1 is reduced in group-housed vs. individually housed pigs, concomitant with a reduction in growth rate and feed intake, and an increase in backfat thickness. These latter findings clearly indicate that energy metabolism and nutrient partitioning are altered by factors as simple as changes in group

size. This interest area has been expanded in Dr. Spurlock's laboratory to encompass the relationships among stress proteins, bacterial endotoxin, and the local production of proinflammatory cytokines in the myocyte as potential causes of the failure of pigs to achieve genetic potential for growth and efficiency in commercial facilities. A recent collaborative arrangement with United Feeds has targeted specific omega-3 fatty acids as regulators of cytokine signaling and growth in both adipocytes and myofibers. Because of access to particular fatty acids through this research relationship, Dr. Spurlock is in a unique position to extend this area of his research and to develop specific nutritional strategies for modulating immune function and growth.

3. **Graduate Research Involvement:**

a. **Completed students:**

1) Kolapo Ajuwon, Regulation of growth and metabolic markers in peripheral tissues of the pig by exogenous leptin, M.S. (Co-advisor with O. Adeola), 2001. (Publication #31, 32, page 6; Abstract #35, page 10).

2) Mahogany Wade, MS. (non-thesis), 2001.

3) Thomas Weber, The effects of exogenous leptin on immunological variables of growing pigs and leptin effects upon cultured monocytes and T-cells, Ph.D., 2003. (Publication #35, page 6; Abstract #37, page 10).

b. **Current students:**

1) Kolapo Ajuwon, Ph.D. (candidate) Immunological roles of the adipocyte and adipocytokines. (Publication #34, 38, page 6; Abstract #41, 42, page 10).

2) Sheila Jacobi, Ph.D. (candidate) Endocrine activity of the porcine adipocyte. (Publication #37, page 6).

3) Sun Hye Shin, Ph.D. (co-advised with Dr. Gerrard). Intercellular communication between adipocytes and myocytes.

c. **Advisory committee member:**

Student	Degree	Major Professor	Dept.	Date of Degree
Chavis, Christy	M.S.	D. Hancock	ANSC	1994
McComb, Molly	M.S.	A.L. Grant	ANSC	1997
Leininger, Michael	M.S.	K. Houseknecht	ANSC	1999
Weber, Thomas	M.S.	A.P. Schinckel	ANSC	2000
McKee, Carrie	M.S.	S. Eicher	ANSC	2001
Velez, Juan Carlos	M.S.	S. Donkin	ANSC	2002
Norberg, Sarah	M.S.	M.A. Latour	ANSC	2002
Durkin, Rachel	M.S.	R. Krisher	ANSC	2003
Crowder, Stacie	M.S.	S.S. Donkin	ANSC	2003
Trapp, Scott	M.S.	B. Richert	ANSC	2003
Herrick, Jason	Ph.D.	R. Krisher	ANSC	2004
Johnson, Trina	M.S.	S. Eicher	ANSC	2004
Williams, Liz	M.S.	Donkin	ANSC	2004
Zhu, Harry	M.S.	D. Moody	ANSC	2004
Dilger, Anna	M.S.	D.E. Gerrard	ANSC	2004
Selig, Kristin	M.S.	A. Bhunia and J. Patterson	ANSC	2004
Arseneau, Jeff	Ph.D.	R.P. Lemenager	ANSC	Current
Follas, Dan	Ph.D.	J. A. Christian	VPB	Current
Hazleton, Sarah	Ph.D.	S. S. Donkin	ANSC	Current
Schreiweiss, Melissa	Ph.D.	P.Y. Hester	ANSC	Current
Cho, Kae Won	Ph.D.	Y.-C. Kim	F&N	Current
Karcher, Darrin	Ph.D.	T. Applegate	ANSC	Current
[1]Camacho, Maria del Carmen	Ph.D.	Rogelio Alonso	Molecular Genetics	Current

[1]Co-advised with Dr. Rogelio Alonso, Department of Molecular Genetics, National Autonomous University, Mexico City, Mexico

4. **Postdoctoral Research Associates:**

1) Shaoquan Ji. 1996-1999. Dr. Ji worked under Dr. Spurlock's direction at Purina Mills, Inc., and is currently a staff scientist at Linco Research, Inc., St. Louis, MO. (Publication #12-14, 17-18, 20, 26, pages 4-5; Abstract #9, 11, 13, 17, 18, 20-22, 30, pages 7-9).

2) Priya Raman. 2000-2002. Dr. Raman had responsibility for a collaborative project with Dr. Donkin that was designed to identify specific pathways in the hepatocyte by which leptin influences energy substrate utilization. She is currently employed at the Indiana University School of Medicine, Diabetes Center, as a Sr. Research Investigator. (Publication #33, page 6; Abstract #34, page 10).

3) Meghan Wulster-Radcliffe. 2003-2004. Dr. Wulster-Radcliffe currently has had responsibility for the macrophage-adiponectin objective in the grant funded by the Biotechnology Research & Development Corp., but is now employed by Eli Lilly & Co. (Publication #36, b1, page 6; Abstract #39, 40, 43, page 10).

4) Nick Gabler. 2004-Present. Dr. Gabler has major responsibility for a joint project with United Feeds, Inc. that involves the regulation of immune response pathways by fatty acids.

5. GRANT ACTIVITIES (Research grants and awards received)

5.1. Current Grants

1. Agency/Title of Grant: Showalter Foundation. A porcine model of the metabolic syndrome.
2. Duration of Funding: 7/1/04 to 6/30/05
3. Total amount of award: $75,000
4. Your role: PI (Co-PI's Drs. Moody and HogenEsch)
5. If Co-PI, for how much of the total funding are you directly responsible: $60,000

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1. Agency/Title of Grant: United States Department of Agriculture – National Research Initiative. Regulation of lipogenesis and lipolysis in porcine adipocytes by leptin.
2. Duration of Funding: 9/15/01 to 9/30/04
3. Total amount of award: $175,000
4. Your role: PI
5. If Co-PI, for how much of the total funding are you directly responsible:

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1. Agency/Title of Grant: United States Department of Agriculture – National Research Initiative. Regulation of lipogenesis and fatty acid oxidation in the pig by adiponectin.
2. Duration of Funding: 10/1/03 to 9/30/06
3. Total amount of award: $240,000
4. Your role: PI
5. If Co-PI, for how much of the total funding are you directly responsible:

5.2. Pending Grants

1.	Agency/Title of Grant:	United State Department of Agriculture – National Research Initiative. Regulation of cytokine production and inflammation in porcine adipocytes by adiponectin and the ppar transcription factors.
2.	Duration of Funding:	1/1/05 to 12/31/07
3.	Total amount of award:	$260,251
4.	Your role:	PI
5.	If Co-PI, for how much of the total funding are you directly responsible:	

1.	Agency/Title of Grant:	National Institutes of Health. Characterizing a new pig model of the metabolic syndrome.
2.	Duration of Funding:	1/1/05 to 12/31/09
3.	Total amount of award:	$1,879,199
4.	Your role:	PI, Co-PIs are Drs. Moody and Gerrard, Animal Sciences, Mittal, Veterinary Pathobiology and Sturek, IU School of Medicine
5.	If Co-PI, for how much of the total funding are you directly responsible:	$964,700

1.	Agency/Title of Grant:	American Diabetes Association. Obesity-related changes in inflammatory status and adiponectin receptors in adipocytes and macrophages, and potential links to the metabolic syndrome.
2.	Duration of Funding:	1/1/05 to 12/31/07
3.	Total amount of award:	$300,000
4.	Your role:	PI
5.	If Co-PI, for how much of the total funding are you directly responsible:	

5.3. Past Grants

1.	Agency/Title of Grant:	Monsanto-Dekalb, St. Louis, MO. Potential biological and molecular indicators of growth and body composition in two commercial sire lines.
2.	Duration of Funding:	4/1/00 to 6/30/02
3.	Total amount of award:	$174,300
4.	Your role:	PI
5.	If Co-PI, for how much of the total funding are you directly responsible:	

1.	Agency/Title of Grant:	Monsanto-Dekalb, St. Louis, MO. Growth and efficiency of two commercial sire lines.
2.	Duration of Funding:	5/15/00 to 11/30/00
3.	Total amount of award:	$32,303
4.	Your role:	Co-PI with Drs. Richert and Schinckel
5.	If Co-PI, for how much of the total funding are you directly responsible:	$6,000

1.	Agency/Title of Grant:	Purdue Agricultural Research Programs Assistantship. Metabolic and immunological responses to bacterial infection: a comparison of genotypes.
2.	Duration of Funding:	1/1/00 to 12/31/02
3.	Total amount of award:	$30,000
4.	Your role:	PI
5.	If Co-PI, for how much of the total funding are you directly responsible:	

1.	Agency/Title of Grant:	Fats and Protein Research Foundation, Inc. Evaluation of the effects of dietary fat, ractopamine and conjugated linoleic acid (cla) on growth performance and pork quality in genetically learn gilts.
2.	Duration of Funding:	9/1/00 to 6/30/02
3.	Total amount of award:	$39,332
4.	Your role:	Co-PI with Drs. Schinckel, Richert, Forrest and Dr. John Eggert, Continental Grain, Inc.
5.	If Co-PI, for how much of the total funding are you directly responsible:	$1,100

1.	Agency/Title of Grant:	Purdue Research Foundation Graduate Fellowship. The importance of adiponectin and leptin to the innate immune response in the pig.
2.	Duration of Funding:	1/1/02 to 12/31/03
3.	Total amount of award:	$25,292
4.	Your role:	PI
5.	If Co-PI, for how much of the total funding are you directly responsible:	

1.	Agency/Title of Grant:	Showalter Foundation. Functional genomics for animal research.
2.	Duration of Funding:	7/1/01 to 6/30/02
3.	Total amount of award:	$83,000
4.	Your role:	Co-PI with Drs. Bidwell and Moody
5.	If Co-PI, for how much of the total funding are you directly responsible:	$21,000

1.	Agency/Title of Grant:	Biotechnology Research and Development Corporation. Adiponectin: a novel regulator of immune function and energy metabolism.
2.	Duration of Funding:	1/1/02 to 4/30/03
3.	Total amount of award:	$59,451
4.	Your role:	PI
5.	If Co-PI, for how much of the total funding are you directly responsible:	

1.	Agency/Title of Grant:	Biotechnology Research and Development Corporation. Regulation of inflammation by a novel adipocyte protein.
2.	Duration of Funding:	1/1/02 to 12/31/03
3.	Total amount of award:	$269,000
4.	Your role:	PI
5.	If Co-PI, for how much of the total funding are you directly responsible:	

Summary of Funding

Year	Total Amount of Grant	Amount of Grant to Dr. Spurlock	Gift in Kind	Total Amount to Dr. Spurlock & Purdue University
2000	$275,935	$194,500	$30,000	$224,500
2001	$258,000	$196,000	$0	$196,000
2002	$353,743	$84,743	$5,750	$90,493
2003	$240,000	$240,000	$8,800	$248,800
2004	$75,000	$60,000	$4,300	$64,300
Total	$1,202,678	$775,243	$48,850	$824,093

b. Gifts:

1) Pig Improvement Co., $30,000. 2000.

2) Nestle-Purina, $18,850. 2002, 2003, 2004.

4) United Feeds, Inc. $45,000. 2004.

6. Agriculture Research Programs (ARP) Project:

ARP Project 51066, Endocrine actions of adipocytes in the control of energy balance and immune function.

7. Evidence of Interdisciplinary Activity:

a. Collaborative effort with Dr. Chris Bidwell to produce leptin research reagents to support metabolic and regulatory studies. Current work includes the development of cell populations for SAGE analysis. Four manuscripts (#11, 13, 14, 25, pages 4-5).

b. Collaborative effort with Dr. Shawn Donkin to identify the signaling pathways by which leptin controls energy metabolism in isolated pig hepatocytes. One manuscript (# 33, page 6).

c. Collaborative effort with Dr. Rebecca Krisher to explore the role of leptin and the leptin receptor in oocyte maturation and embryonic development, pre-implantation.

d. Collaboration with Dr. Diane Moody to test the efficacy of human microarrays for pig tissues. Models include *in vivo* and *in vitro* exposure of adipocytes to leptin and lipopolysaccharide. Two Showalter grants funded, NIH and 21st Century grants pending.

e. Collaboration with Dr. Karen Houseknecht to investigate the relationship among dietary fatty acid profile, leptin, and peroxisome proliferator activated receptors. Five manuscripts (#19, 22, 23, 25, 28, page 5) and five abstracts (#25, 28, 29, 31, 32, page 9).

f. Collaborative studies with Dr. Alan Grant in the regulation of IGF-1 during immune challenge and stress, and in response to leptin. One manuscript (#14, page 4) and three abstracts (#6, 14, 15, pages 7-8).

g. Collaboration with Drs. Dave Gerrard and Scott Mills to establish *beta*-adrenoceptor density in porcine oxidative and glycolytic muscle, and the influence of ractopamine on these receptors. Also collaborated with Dr. Gerrard to show that myostatin mRNA was localized to the myofiber and secretory epithelial cell in mammary gland. One manuscript (#17, page 4) and one abstract (#6, page 6); NIH grant pending.

h. Collaboration with Dr. Scott Mills pertaining to the regulation of lipogenic genes by dietary fat and growth hormone, and regulation of lipolysis by ß-adrenoceptor agonists. One manuscript (#29, page 5).

i. Collaboration with Dr. Rex Gaskins, University of Illinois. Past projects have been designed to help understand the immunological role of the small intestine in the weanling pig. One manuscript (#21, page 5).

j. Collaboration with Dr. Bob Matteri, USDA-ARS, to study the regulation of the leptin receptor in porcine adipose tissue and skeletal muscle. Dr. Matteri has accepted a new position within the USDA-ARS, and Dr. Spurlock has assumed full responsibility for this project.

k. Collaborative project with Dr. Jess Miner, University of Nebraska, to identify the regulatory roles of adipsin, with an emphasis on the adipocyte. Two manuscripts (#8, 27, pages 4 & 5).

l. Collaboration with Dr. John Christian, Purdue University School of Veterinary Medicine, to establish procedures for deriving functional macrophages from peripheral blood monocytes. One manuscript (#36, page 6) and one abstract (#39, page 10).

m. Collaboration with Dr. Merv Yoder, Indiana University School of Medicine, to establish a co-culture system for adipocytes and myofibers using a pluripotent cell of muscle origin.

n. Collaboration with Dr. Suresh Mittal, Purdue University School of Veterinary Medicine, to use adenovirus technology to study adiponectin in pigs (USDA-NRI funded, 2003; NIH grant pending).

o. Collaboration with Dr. Rogelio Alonso, Dept. of Molecular Genetics, National Autonomous University of Mexico (UNAM), Mexico City, Mexico. This project entails a physiologic and genetic comparison of the Mexican Hairless Pig and the Cuino as potential models of obesity and Type II diabetes. Dr. Spurlock has been admitted to the graduate faculty at UNAM, and he and Dr. Alonso are co-advising a Ph.D. student on this project.

p. Collaboration with Dr. Anne Reifel-Miller, Eli Lilly & Co. Dr. Reifel-Miller has provided PPAR ligands and adiponectin receptor adenoviral constructs, and will be providing assays for a pending Diabetes grant.

q. Collaboration with Dr. Mike Sturek, Indiana University School of Medicine, to develop the Ossabaw pig as a model for the metabolic syndrome. 21st Century and NIH grants pending.

8. Other Evidence of Recognition:

a. Peer Review Contributions to Scientific Journals:

1) Member of editorial board, *Journal of Animal Science*-Nonruminant Nutrition (1998-2001).

2) Member of editorial board, *Domestic Animal Endocrinology* (2000-2003).

3) School of Agriculture Nominee, David and Lucille Packard Research Fellowship Competition (2003).

4) Invited contributor, *Nutrition Reviews International.*

5) Member of editorial board, *Journal of Animal Science*-Growth and Development (2004-2007).

6) *Ad hoc* reviewer for the following journals:
   a) *American Journal of Physiology*
   b) *Journal of Interferon & Cytokine Research*
   c) *Hepatology*
   d) *Physiology & Behavior*
   e) *Nutritional Biochemistry*
   f) *Journal of Nutrition*
   g) *Journal of Endocrinology*

b. Grant Review Activity:

Dr. Spurlock serves as an ad hoc reviewer for the United States Department of Agriculture National Research Initiative Competitive Grants Program. Since 1996, he has reviewed an average of five proposals per year for the Growth and Development, Genetic Mechanisms and Animal Health and Well-Being divisions. Dr. Spurlock also serves as an ad hoc reviewer for the National Institutes of Health, Adipocyte Biology and NIDDK sections, and for the Veterans Administration, Gerontology section.

c. Contributions to American Society of Animal Science:

1) Midwest section, member of program committees
   Nonruminant Nutrition (1996-1998).
   Growth and Development (1999-2001; Chair, 2001).

2) Midwest section meetings, scientific session chair in 1996, 1997, 1998 and 1999.

3) National ASAS meetings, scientific session chair in 1996, 1998, and 1999.

4) National ASAS Triennial Growth Symposium Committee (2003-2004).

d. Advisory Board Memberships:

1) Monsanto-DeKalb; appointed to team focused on improving the efficiency of feed utilization in commercial genetic lines (2000-2003).

2) Pig Improvement Company (PIC); appointed to research advisory board charged with designing the research strategy to increase the realization of genetic potential in PIC genetic lines (1999-2003).

3) AusGene; member of an academic consulting team led by Dr. Terry Stewart to address issues related to the optimal selection of genetically superior animals (2000 to present).

4) Appointed *ad hoc* member, Research Advisory Board, School of Veterinary Medicine, Purdue University (2002-2005).

e. Faculty Mentoring:

1) Dr. Spurlock is currently acting as a mentor in support of a K01 grant for Dr. Ignacio Camarillo, Dept. of Biology, Purdue University. This is an NIH grant that requires joint laboratory activities between Drs. Spurlock and Camarillo, and development of an integrated project. This grant targets the relationship between adipocytes and mammary epithelial cells in mammary tumorigenesis.

B. EXCELLENCE IN TEACHING

Even though Dr. Spurlock does not have a primary teaching appointment, he taught ANSC 324 (Animal Nutrition), a very important course in the curriculum, for two years. This is one of three courses that will fulfill the advanced nutrition requirement for Animal Sciences majors and typically has an enrollment of 85-100 students. Dr. Spurlock's approach to this course, and to teaching in general, was to present the facts, develop the concepts, and then guide the students through the practical applications of the knowledge. The ANSC 324 course emphasizes the application of nutrition principles to the formulation and preparation of diets that adequately meet the nutrient needs of animals. Considerable effort is devoted to helping students grasp the fundamentals of computerized diet formulation, using software that is common to the commercial feed and production industries. Laboratory assignments and homework problems are used to cultivate nutritional expertise. The students are challenged to apply their knowledge to carry out a research project in which they work individually and in teams to formulate and mix diets to accomplish an assigned nutrition objective. The students feed and care for their chickens for a period of two weeks. Data are collected, analyzed statistically, and reported in journal article (Poultry Science Journal or Journal of Nutrition) format.

Dr. Spurlock also introduced two new focal points for the course. First, two laboratory periods are now devoted to the practice of feed microscopy. Students are taught to recognize specific feed ingredients via their inherent microscopic characteristics, and are instructed as to the potential uses and limitations of feed microscopy from a production and regulatory perspective. Secondly, one week (lecture and laboratory) is now devoted to companion animal nutrition. Dr. Spurlock collaborated with research and technical service scientists from the Ralston Purina Company to conduct the sessions that identified unique nutrient needs of companion animals, in addition to reading and understanding pet food labels. Also, concepts of prophylactic and therapeutic nutrition were introduced in conjunction with the potential for specific nutrients and feed ingredients to impact physiological processes.

Dr. Spurlock has also developed an adipocyte biology section for the new course, FN/ANSC 595. This module has been taught for two years, and has led Dr. Spurlock to develop and teach a new graduate level adipocyte biology course (ANSC 595R) that covers basic aspects of energy metabolism in adipocytes, but focuses largely on the newly discovered role of the adipocyte as an endocrine and immune cell. This course was initiated in the Fall semester, 2003. Special emphasis is placed on biochemical pathways relating to energy utilization and regulation of immune function in

light of the hormones, growth factors, and cytokines produced by the adipocyte, and the tissues bearing receptors to these regulatory factors. The initial evaluation of this course by graduate students was excellent. Dr. Spurlock will teach this course for the second time in the Fall of 2004.

1. Courses taught:

Course	Semester, year	Credits	Number of Students
ANSC 324	Spring, 2001, 2002	3	87
ANSC 595R	Fall, 2003	2	12

2. Student evaluations:

Course	Year	No. of Responses	University Core[a]		Departmental Core[b]					
			1	2	1	2	3	4	5	6
ANSC 324	2001	74	4.2	4.7	4.7	4.7	4.4	4.2	4.1	4.3
ANSC 324	2002	73	4.0	4.6	4.3	4.6	3.9	4.0	4.1	4.2
ANSC 595R	2003		4.7	4.9	4.9	5.0	4.6	4.9	5.0	4.8

[a]University Core Questions (strongly agree=5):
Overall, I would rate this course as Excellent-Good-Poor-Very Poor
Overall, I would rate this instructor as Excellent-Good-Poor-Very Poor

[b]Departmental Core
1. My instructor seems well prepared for class.
2. Students are encouraged to see the instructor if they are having difficulty.
3. My instructor gives exams, which accurately reflect the course material.
4. The climate of this class is conductive to learning.
5. This course effectively challenges me to think.
6. This course builds understanding of concepts and principles.

C. EXCELLENCE IN EXTENSION AND SERVICE

Dr. Spurlock does not have a formal extension appointment, but participates in activities as requested. He was a speaker at Purdue's 80th annual Swine Day (2000) and has participated on several committees.

1) Service on School Committees:

a. Roadmapping and Strategic Planning Committee, 2002
b. Technology transfer subcommittee, 2001
c. Relationship development, Purdue and Dow AgroSciences, 2003 to present
d. Agriculture Life Sciences Summit, 2004
e. Dean of Agriculture Search Committee, 2004

2) Service on Departmental Committees:

a. Meats Laboratory and Facilities Committee, 1999-2002
b. Renovation of Lilly Animal Facility Committee, 2000
c. Louja Graduate Student Travel Award Committee, 2000
d. Swine Programs Committee, 2000
e. Swine Day Planning Committee, 2000
f. Chair, Growth and Development Committee for Cooperative State Research, Education and Extension Service (CSREES) Review, 2001
g. Chair, Nutrient Utilization Search Committee, 2001
h. Department Head Search Committee, 2001
i. Graduate Committee, 2001-2003
j. Interdepartmental Nutrition Programs Admissions Committee, 2001-2003
k. Advisor, Block & Bridle Club, 2001-2003
l. Chair, Research Strategic Planning Team, 2002-2003
m. Chair, Departmental Seminar Committee, 2002

3) Professional Improvement Activities:

a. International conference and workshop on proteases, Tokyo, Japan, 1994
b. Growth biology techniques workshops, Minneapolis, MN, 1994
c. University of Florida-USDA grantmanship workshop, Orlando, FL, 1999
d. "Writing a Successful NIH or USDA Grant" workshop, Purdue University, West Lafayette, IN, 2000
e. ESCOP/ACOP Leadership Program (Class 13, Phase), West Lafayette, IN; Indianapolis, IN; Washington, DC. 2003-2004

SUMMARY OF SUPPORTING STRENGTHS

		Page(s)
I.	Evidence of Excellence in Research	
	A. Developed a focused research program to uncover strategies to improve the efficiency of meat animal production.	2, 14-17
	B. Recipient of the School of Agriculture Research Award.	2
	B. Authored or co-authored 41 manuscripts in 13 different peer-reviewed journals, 5 manuscripts in conference proceedings and 43 published abstracts.	3-11
	C. Awarded two patents, with a third pending.	13
	D. Obtained $1,202,678 (directly responsible for $824,000) in research support.	19-23
	E. Currently has three Ph.D. students and has guided three post-doctoral scientists.	17-18
	F. Collaborates effectively with several other scientists.	24-25
II.	Evidence of Excellence in Teaching	
	A. Received excellent student evaluations in Animal Nutrition (ANSC 324) and Adipocyte Biology (ANSC 595R).	28
	B. Participates in an adipocyte module for course for Animal Sciences and Foods & Nutrition graduate students, and has developed a graduate level adipocyte biology course.	27
III.	Evidence of National and International Recognition	
	A. Twenty-eight invited research talks in domestic and international forums.	11-13
	B. Member of editorial boards of two prestigious journals concurrently.	25
	C. Serves as ad-hoc reviewer for seven additional scientific journals.	26
	D. Serves as an ad-hoc reviewer for three divisions of the USDA-NRICGP, two sections of the NIH, and one section of the Veterans Administration.	26
	E. Is (or has been) a member of four advisory boards.	26-27
	F. Invited to graduate faculty at UNAM; invited collaboration on government research project to compare the metabolic and genetic aspects of two potential pig models of obesity and diabetes.	25

SUPPORTING LETTERS – DR. MICHAEL E. SPURLOCK

- **Dr. Cliff Baile** holds the titles of Distinguished Professor of Animal Sciences and Foods and Nutrition and Eminent Scholar in Agricultural Biotechnology at the University of Georgia.

  Dr. Baile is acquainted with Dr. Spurlock's research through professional and collaborative interactions while Dr. Spurlock was at Purina Mills, Inc., and at Purdue University

- **Dr. Rodney Johnson** is currently Professor, Integrative Biology, Department of Animal Sciences, University of Illinois.

  Dr. Johnson is a world renowned expert in the relationship between growth and the immune system. His laboratory has done pioneering work to define the regulatory linkages between disease and the molecular regulation of cytokine production within the central nervous system. Dr. Johnson and Dr. Spurlock have been joint speakers at multiple conferences over the past 10 years.

- **Dr. Jack Odle** is currently Professor of Nutrition, Department of Animal Sciences, North Carolina State University.

  Dr. Odle's program is focused in the area of neonatal nutrition and metabolic regulation. Emphasis is on developmental aspects of lipid digestion, absorption and metabolism at the molecular, cellular and whole-animal level. Dr. Odle is an extremely well-published scientist, and has served as the associate editor for the Journal of Nutrition.

- **Dr. Karen Houseknecht** is currently a Sr. Research Leader at Pfizer's Central Research Division in Groton, Connecticut.

  She has responsibility for the group that leads the company's research efforts in the phenotypes common to the metabolic syndrome. She has collaborated extensively with Dr. Spurlock and her expertise in metabolic diseases is widely recognized.

- **Dr. Harm HogenEsch** is currently Professor and Head, Department of Veterinary Pathobiology, School of Veterinary Medicine, Purdue University, and is also the director of the Center of Excellence in Comparative Medicine at Purdue University.

  Dr. HogenEsch has served on doctoral student committees with Dr. Spurlock, and has had numerous interactions via the comparative medicine program.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Michael E. Spurlock	
Appln. No.	:	09/928,522	
Filed	:	August 13, 2001	Group Art Unit: 1647
Title	:	Bovine Leptin Protein, Antisense and Antibody	Examiner: C. J. Saoud
Docket No.	:	LL31.12-0015	

DECLARATION UNDER 37 C.F.R. § 1.132

MS Fee Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

SENT VIA EXPRESS MAIL

Express Mail No.: EV 485710565 US

Sir:

I, Michael E. Spurlock of 10254 East 200 North in Lafayette, IN 47905, hereby declare as follows:

1. I am currently an Associate Professor in the Department of Animal Sciences at Purdue University in West Lafayette, Indiana.

2. From 1993 to 1999, I was employed with Purina Mills, Inc. initially as a Research Scientist, later as Senior Research Scientist and then as a Research Manager, and ultimately as Senior Research Manager.

3. I earned a Bachelor of Science degree in Animal Science from the University of Missouri in Columbia, Missouri in 1981.

4. I earned a Master of Science degree in Nutritional Biochemistry from the University of Missouri in Columbia, Missouri in 1987.

5. I earned a Doctor of Philosophy (PhD) degree in Nutritional Biochemistry (with minors in histology and cell biology) from the University of Missouri in Columbia, Missouri in 1989.

6. From 1991 to 1993, after completing my PhD, I was a Postdoctoral Research Fellow in the Department of Animal Sciences at Purdue University where I researched ß-adrenoceptor kinetics and also researched signal transduction in adipose tissue and skeletal muscle.

7. A brief resume of my professional career from 1989 to the present is attached hereto as Exhibit A.

8. I have authored (or coauthored) more than thirty-five published articles, including topics concerning leptin technology; many of these published articles are listed in my resume of Exhibit A. Some of my published leptin articles are listed below:

* Houseknecht, K.L., and M.E. Spurlock. 2003. The Regulation of Lipid Metabolism in Peripheral Tissues by Leptin, Invited Review, Nutrition Reviews International 16:83-96;

* Ji, S.Q., Scott, R.R., Willis, G.M., and Spurlock, M.E. 1998. Partial Cloning and Expression of the Bovine Leptin Gene, Anim. Biotech. 9:1-14;

* Houseknecht, K.L., Baile, C.A., Matteri, R.L., and Spurlock, M.E. 1998. The Biology of Leptin: A Review, J. Anim. Sci. 76:1405-1420;

* Houseknecht, K.L., Portocarrero, C.P., Ji, S.Q., Lemenager, R., and Spurlock, M.E. 2000. Growth Hormone Regulates Leptin Gene Expression In Bovine Adipose Tissue: Correlation With Adipose IGF-1 Expression, J. Endocrinol. 164:51-57;

* Houseknecht, K.L. and Spurlock, M.E.. 2003. The Regulation of Lipid Metabolism in Peripheral Tissues By Leptin, Nutri. Rev. Intl. 16:83-96;

* Wulster-Radcliffe, M. C., J. A. Christian, J. Wang, and M. E. Spurlock. 2004. The Anti-inflammatory Actions of Adiponectin Include the Regulation of Il-6 and Il-10, and a Suppression of Cell Proliferation That Is Associated with Increased Caspase Activity, Biochem. Biophys. Res. Comm. 316:924-929.

9. Also, I presented have been invited to deliver more than twenty-five research presentations in the U.S. and internationally on microbiologic topics including topics concerning leptin technology; many of my research presentations are listed in my resume of Exhibit A. Some of my more research presentations concerning leptin are listed below:

* Leptin: A Role in Food Animal Production?; University of Nebraska; Lincoln, Nebraska; 1997;

* The Cytokinology of Leptin, Amer. Soc. Of Anim. Sci. Leptin Symp.; Nashville, Tennessee; 1997;

* The Status of Leptin and Myostatin in Food Animal Biology, Pfizer Animal Health Symp.; Groton, Connecticut; 1999;

* Linking Energy Balance to Immune Function Through Leptin and Adiponectin, Brit.. Soc. Of Anim. Sci. Symp.; York, England; 2002.

10. I am experienced in isolating, purifying, and manipulating DNA sequences, such as bovine, porcine, murine, and human DNA sequences and am also skilled in experimental procedures of leptin genetics, such as techniques for establishing the impact of leptin on lipid accumulation via processes including lipolysis and lipogenesis, due to my education and work experience relating to DNA sequence and leptin genetics issues over the past twenty-five years.

11. I am an inventor of the invention described and claimed in U.S. Patent Application Serial No. 09/928,522 filed on August 13, 2001 and am an inventor of the invention defined and claimed in U.S. Serial Application No. 08/688,908, now U.S. Patent No. 6,297,027.

12. The formation of a duplex by nucleic acid hybridization (base pairing between two nucleic acid molecules) is directly related to the degree of stringency of the hybridization conditions employed. Rawn, J. David, Biochemistry, Pages 993-994 (Carolina Biological Supply Company, 1989) (attached as Exhibit B of my Declaration).

13. Persons of ordinary skill in the art of molecular biology know high temperature is an example of a stringent hybridization condition:

> Both a temperature too low or an ionic strength to high will reduce the stringency of hybridization and may negatively affect the specificity of the detected signal. In contrast, raising the temperature, decreasing the ionic strength or both, will increase stringency.

Neumaier, M., Braun, A., and Wagener, C., 1998, <u>Fundamentals of Quality Assessment of Molecular Amplification Methods in Clinical Diagnostics</u>, <u>Clinical Chemistry</u>, 44:12-26 (attached as Exhibit C).

14. Persons of ordinary skill in the art of molecular biology know another example of a stringent hybridization condition is the chemical composition of the hybridization solution, such as hybridization solution with relatively low ionic strength (i.e. a relatively low salt concentration). See page 993 of attached Exhibit B. See also Exhibit C (see recited passage in Paragraph 13 above from Exhibit C).

15. Thus, based on the statements of Paragraphs 13 and 14, persons of ordinary skill in the art of molecular biology know the stringency of hybridization conditions may be adjusted by varying the temperature at which hybridization is performed, the chemical composition of the hybridization solution used during hybridization experiments, or both the hybridization temperature and the chemical composition of the hybridization solution.

16. Persons of ordinary skill in the art of molecular biology know time of exposure to hybridization solution at a particular temperature may also be manipulated to attain stringent hybridization conditions; for example, hybridization overnight for a time ranging from twelve to sixteen hours is generally sufficient for most probes and blots to complete base pairing between a nucleic acid sequence and a DNA or mRNA sequence:

> Generally, hybridizations are carried on overnight for 12 to 16 hr. This is sufficient for most probes and blots.

Hybridization with Radioactive Probes: Using DNA Fragments as Probes. Current Protocols in Molecular Biology. Section II 6.3.5. 2000. (attached as Exhibit D).

17. Persons of ordinary skill in the art of molecular biology also know another technique for improving specificity of base pairing entails incorporation of non-specific DNA in the hybridization solution:

> B. Prehybridization & Hybridization
>
> Since the nylon membrane likes to bind things, background sites are blocked (bound) with non-specific DNA and protein. Usually, sheared salmon sperm DNA is used in prehybe to block these sites.

Instructions posted at http://catlserver.tamu.edu/ning/ing626.htm for Lab Session No. 6, Fall 2003: Course Entitled Gene Expression (ANSC/GENE 626 (edited by. N. Ing 9/2/03)); Nancy H. Ing, Instructor; Texas A & M University; College Station, Texas (attached as Exhibit E). The non-specific DNA is said to prevent the probe from hybridizing nonspecifically - that is with nucleic acid that is not complementary (specific) to the probe nucleic acid:

> A solution commonly used during probe hybridizations that involve filters (such as Southern, Northern, or Western blots). The solution contains ficoll, bovine serum albumin, polyvinylpyrrolidone (PVP), and a high concentration of nonspecific DNA so the probe won't hybridize nonspecifically.

See Hyperdictionary @ www.hyperdictionary.com/dictionary/Denhardt's + solution; obtained from the Internet on November 12, 2004 (attached as Exhibit F).

18. Persons of ordinary skill in the art of molecular biology know one example of non-specific DNA that may be used to block hybridization of non-specific DNA with the probe nucleic acid is salmon sperm. Information page entitled Eppendorf® Sheared Salmon Sperm DNA:

> Sheared Salmon Sperm DNA is used as a blocking agent to reduce the background in hybridization experiments.

Information page entitled Eppendorf® Sheared Salmon Sperm DNA located at http://www.brinkmann.com/product.asp?path=115&ref=136;obtained from the Internet on November 12, 2004 (attached as Exhibit G).

19. Beyond controlling the hybridization stringency directly, by varying the temperature at which hybridization is performed and/or the chemical composition of the hybridization solution used during hybridization experiments (including use of blocking substances as described in Paragraphs 17-18), those of ordinary skill in the art of molecular biology know hybridization stringency may also be controlled by post-hybridization washing conditions:

> You can also control for stringency of hybridization at the next step, which is washing the unbound probe away from the blot. If you wash at high temperature or low salt concentration, you will remove any hybrids that are not perfectly matched.

Instructions posted at http://www.clarkson.edu/class/by412/word/Northern%20hybridization.doc (obtained from the Internet on November 12, 2004) for Molecular Biology Lab #17 (Northern Transfer and Hybridization Experiment #2: Labeling Probe and Hybridization) taught Fall, 2004 at Clarkson University in Pottsdam, New York by Craig Woodworth (attached as Exhibit H).

20. The invention of the above-identified application is, in one aspect, directed to nucleic acid molecules (and functional variants thereof), such as (1) single or double-stranded DNA (and cDNA and genomic DNA) and (2) RNA (and mRNA), that encode bovine polypeptide leptin. (Page 2, line 29, to page 3, line 9; page 5, line 17, to page 6, line 4; page 7, lines 3-28; and page 8, lines 6-26).

21. According to the present invention, the nucleic acid molecules (and functional variants thereof) encoding for bovine leptin polypeptide mentioned in Paragraph 20 hybridize and are capable of hybridizing to nucleotide sequences (and portions thereof), such as various nucleotide sequences (and portions thereof) disclosed in the above-identified application. (Page 3, lines 17-23, and page 14, line 23, to page 15, line 3).

22. In another aspect, the invention of the above-identified application is directed to methods of hybridizing the nucleic acid molecules encoding for bovine leptin polypeptide (and functional variants thereof) mentioned in Paragraph 20 to nucleotide sequences (and portions thereof), such as various nucleotide sequences (and portions thereof) disclosed in the above-identified application. (Page 18, line 25, to page 20, line 7).

23. Example II of the above-identified application provides some particular guidance about hybridization conditions that may be employed when practicing the hybridization methods of the present invention that are the subject of Paragraph 22. This guidance illustrates that stringent hybridization conditions are suitable for practicing the hybridization methods of the present invention.

24. Example II of the above-identified application reads as follows:

Isolation of mRNA Corresponding to Bovine Leptin cDNA

The bovine leptin cDNA was used as a probe for detection of the full length mRNA on a Northern blot containing bovine adipose tissue poly A^+ mRNA and *ob/ob* mouse adipose total RNA (FIG. 7). The RNA samples were separated on a 1% formaldehyde agarose gel and then transferred to a nylon membrane (Zeta-probe, Biorad) by a capillary transfer method in 10xSSC (1.5M NaCl, 0.15 M Sodium Citrate, pH 7.0). The blot was hybridized with an alpha-[^{32}P] dCTP labeled bovine leptin cDNA in hybridization solution (Gibco BRL; 0.9 M NaCl, 0.09 M Sodium Citrate (pH 7.0), 0.01 M EDTA (pH 8.0), 5xDenhart's Solution (0.1% Ficoll, 0.1% polyvinylpyrolidone, 0.1% BSA), 0.5% SDS, 100μg/ml sheared, denatured salmon sperm DNA) at 55C.

for 20 h. The blot was washed to a final stringency of 0.1xSSC (0.015M NaCl, 0.0015 M sodium citrate (pH 7), 0.1% SDS at 60C. and exposed to X-ray film. The bovine leptin mRNA (approximately 3,090 bp) was clearly evident in the bovine adipose tissue and an approximate 3,240 bp leptin mRNA was detected in the *ob/ob* mouse adipose tissue. As shown in FIG. 7, lanes 7-8 contain the *ob/ob* mouse adipose total RNA and lane 10 contains the bovine adipose poly A^+ mRNA.

Abundance of the bovine leptin mRNA was low; therefore, a more sensitive RNAse protection assay (RPA) was established to quantify bovine leptin mRNA in adipose tissue. Briefly, a T7 promoter DNA sequence was added to the antisense leptin *ob* primer via PCR with the sense primer as described in Example I. This modified antisense primer produced a 478 bp fragment containing the T7 promoter. A radiolabeled riboprobe was then generated by in vitro transcription with alpha-[^{32}P]-UTP and the 478 bp PCR fragment. The RPA was performed using a commercially available kit (RPA II, Ambion, Inc.). Hybridization was done with 50,000 cpm of the bovine leptin riboprobe and 10μg of adipose total RNA for 20 h at 42-45C. Single-stranded RNA was then digested by a 1:50 dilution of RNAse T1 for 30 min at 37C. After ethanol precipitation, the protected fragment was separated in a 5% polyacrylamide gel with 8M urea. The gel was then dried and exposed to X-ray film and a single 449 bp fragment was protected. Beta-actin was used as an internal control for standardization of the RPA results.

25. Thus, Example II discloses a hybridization trial using bovine leptin cDNA as a probe to detect full length bovine leptin mRNA.

26. According to Example II, bovine leptin cDNA was hybridized against bovine leptin mRNA at 55°C for twenty hours.

27. According to an industry source, a hybridization temperature of about 50°C is generally sufficient to establish stringent conditions (specifically, 50°C in numbered para. 4 on the first page of Exhibit I, 55°C in numbered para. 4 on the second page of Exhibit I, and 50°C in numbered para. 4 on the third page of Exhibit I) is adequate to produce stringent conditions using a non-formamide hybridization solution. Connolly, Amy L. and Jones, Teri L.,

Hybridization Buffers I: Comparison of Formamide vs. Aqueous Hybridization Solutions, four pages (KPL Research & Development August, 2002) (attached as Exhibit I). See page 13, lines 6-7, of U.S. Patent No. 6,156,546 of Exhibit J that references use of formamide to enhance hybridization stringency.

28. The evidence provided in Paragraphs 26-27 regarding use of a hybridization temperature of 55°C during the hybridization trial of Example II of the present application illustrates, to those of ordinary skill in the art of molecular biology, use of stringent hybridization conditions during the hybridization trial of Example II. This statement is probative as to stringency even if the salt concentration in the hybridization solution of Example II is not taken into account. (See Paragraph 15 above).

29. Next, we consider the hybridization time of twenty hours employed in Example II. See Paragraphs 24 and 26 above.

30. Hybridization overnight for a time ranging from twelve to sixteen hours is generally sufficient for most probes and blots to complete base pairing between a nucleic acid sequence and a DNA or mRNA sequence:

> Generally, hybridizations are carried on overnight for 12 to 16 hr. This is sufficient for most probes and blots.

See page 3 of Exhibit D

31. Others in the molecular biology industry indicate hybridization overnight (specifically, numbered para. 4 on the first page of Exhibit I, numbered para. 4 on the second page of Exhibit I, and numbered para. 4 on the third page of Exhibit I) is adequate to produce stringent conditions using a non-formamide hybridization solution. See Exhibit I.

32. The evidence provided in Paragraphs 29-31 regarding hybridization for twenty hours, which clearly qualifies as at least overnight, during the hybridization trial of Example II of the present application illustrates, to those of ordinary skill in the art of molecular biology, use of stringent hybridization conditions during the hybridization trial of Example II.

33. Next, we consider the salt concentration employed in the hybridization solution of Example II. Specifically, since the hybridization solution contained 0.9 M NaCl and 0.09 M sodium citrate, one of ordinary skill in the art of molecular biology would understand the hybridization solution contained 0.99 M sodium ion. See Paragraph 24 above.

34. Those of ordinary skill in the art of molecular biology know salt concentrations of 0.99 M sodium ion are considered to be low salt concentrations generally sufficient to establish stringent conditions:

> Typically, stringent conditions will be those in which the salt concentration is less than about 1.0 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7. 0 to 8.3 . . . .

Page 12, lines 2-4, of WO 02/036829A2 (attached as Exhibit K).

35. The evidence provided in Paragraphs 33 and 34 regarding use of a hybridization solution with a salt concentration of 0.99 M sodium ion during the hybridization trial of Example II of the present application illustrates, to those of ordinary skill in the art of molecular biology, use of stringent hybridization conditions during the hybridization trial of Example II. This statement is probative as to stringency even if the hybridization temperature of Example II is not taken into account. (See Paragraph 15 above).

36. Next, we consider the salmon sperm concentration of 100 μg/ml that is employed in the hybridization solution of Example II. See Paragraph 24 above.

37. Persons of ordinary skill in the art of molecular biology also know another technique for improving specificity of base pairing entails incorporation of non-specific DNA in the hybridization solution. See Exhibit E, as discussed in Paragraph 17 above. The non-specific DNA is said to prevent the probe from hybridizing nonspecifically - that is with nucleic acid that is not complementary (specific) to the probe nucleic acid. See Exhibit F, as discussed at Paragraph 17 above.

38. Salmon sperm at a concentration of 100 μg/ml is incorporated by those skilled in the art of molecular biology to complement and support high stringency hybridization by limiting non-specific nucleic acid binding during hybridization:

> In a most preferred embodiment, a nucleic acid sequence that is hybridizable to a nucleic acid sequence (or a complement of the foregoing) encoding the aforementioned peptides, or a derivative of the same, under conditions of high stringency is provided. By way of example and not limitation, procedures using such conditions of high stringency are as follows: Step 1: Filters containing DNA are pretreated for 8 hours to overnight at 65°C in buffer composed of 6× SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500 µg/ml denatured salmon sperm DNA. Step 2: Filters are hybridized for 48 hours at 65°C in the above prehybridization mixture to which is added 100 mg/ml denatured salmon sperm DNA and 5-20××10^6 cpm of ^{32}P-labeled probe.

Column 14, lines 33-47, of US Patent No. 6,777,388 (attached as Exhibit L).

39. The evidence provided in Paragraphs 36-38 regarding use of a hybridization solution with a salmon sperm concentration of 100 μg/ml during the hybridization trial of Example II of the present application illustrates, to those of ordinary skill in the art of molecular biology, use of conditions that inhibit non-specific probe hybridization and therefore further support attainment of results consistent with use of stringent conditions during the hybridization trial of Example II.

40. We next consider the post-hybridization washing conditions of Example II that entailed washing "to a final stringency of 0.1xSSC (0.015 M NaCl, 0.0015 M sodium citrate), 0.1% SDS at 60°C. See Paragraph 24 above.

41. As noted above, beyond controlling the hybridization stringency directly, by varying the temperature at which hybridization is performed and/or the chemical composition of the hybridization solution used during hybridization experiments , those of ordinary skill in the art of molecular biology know hybridization stringency may also be controlled by post-hybridization washing conditions, such as the temperature and/or the salt concentration. See Paragraph 19 above. For example:

> You can also control for stringency of hybridization at the next step, which is washing the unbound probe away from the blot. If you wash at high temperature or low salt concentration, you will remove any hybrids that are not perfectly matched.

See Exhibit H.

42. In this regard, those of ordinary skill in the art recognize that high stringency washing may be accomplished using the hybridization temperature in combination with a final washing solution containing 0.1 x SSC and 0.1% SDS, as was employed in Example II:

> Following hybridisation, high stringency washing may be done in several steps, with a final wash (about 30 min) at the hybridisation temperature in 0.2-0.1 times SSC, 0.1% SDS.

Paragraph 0188 of US20040137492A1 (attached as Exhibit M).

43. The evidence provided in Paragraphs 40-42 demonstrates those of ordinary skill in the art of molecular biology recognize that high stringency washing may be accomplished using a final washing solution containing 0.1x SSC and 0.1% SDS, as was employed in Example II, and also demonstrates this stringent washing solution may be employed at a temperature at least as high as the hybridization temperature to further enhance the washing stringency.

44. The facts presented in Paragraphs 23-43, as supplemented by Exhibits B-M of my Declaration, illustrate that one of ordinary skill in the art, upon reviewing the hybridization conditions employed in Example II of the above-identified application, would recognize these hybridization conditions as being stringent hybridization conditions that would typically enable a probe, such as the bovine leptin cDNA molecule employed in Example II, to hybridize to a perfect or near perfect nucleic acid molecule complement of the probe.

45. Example III of the above-identified application also provides some particular guidance about hybridization conditions that may be employed when practicing the hybridization methods of the present invention that are the subject of Paragraph 22. This guidance further illustrates that stringent hybridization conditions are suitable for practicing the hybridization methods of the present invention.

46. Example III of the above-identified application reads as follows:

> ISOLATION OF GENOMIC DNA CLONE CORRESPONDING TO BOVINE LEPTIN
>
> The bovine leptin cDNA was also used to screen a bovine genomic DNA library. Specifically, a bovine genomic library (Holstein dairy cow) was purchased from a commercial source (Stratagene, Inc.). The library, containing $2x10^6$ plaque forming units (pfu) before amplification, was constructed in lambda FIX II vector with insert sizes of 9-23 kb. Procedures for genomic library screening were those recommended by the supplier. About $1.2x10^6$ pfu were screened in the primary screening plates. Specifically, two sets of replica nylon filters were lifted from plates and prehybridized for 3 h at 40-42C. in 0.8 M NaCl, 0.02 M pipes (pH 6.5), 50% formamide, 0.5% SDS, and 100 μg/ml denatured, sonicated salmon sperm DNA. Filters were hybridized overnight with [alpha-^{32}P] dCTP labeled bovine leptin cDNA probe in hybridization buffer with the same composition as the prehybridization solution for 21 h. Filters were subsequently washed with a final stringency of 0.1xSSC, 0.1% SDS at 60C. for 30 min. After exposure to X-ray film, positive clones that showed signals on both replica filters were recovered from the agar plates, retitered and tested in secondary

> and tertiary screening using the same protocol. After three rounds of screening, four individual positive clones were identified for further use.

46. Thus, Example III discloses a hybridization trial that used bovine leptin cDNA as a probe to screen a bovine genomic DNA library and detect bovine leptin DNA.

47. According to Example III, the bovine leptin cDNA was hybridized against the bovine leptin DNA for twenty-one hours.

48. Hybridization from 12 hours to 16 hours is generally recognized by those of ordinary skill in the art of molecular biology as being sufficient for most probes and blots to complete base pairing between a nucleic acid sequence and a DNA sequence:

> Generally, hybridizations are carried on overnight for 12 to 16 hr. This is sufficient for most probes and blots.

See page 3 of Exhibit D.

49. The evidence provided in Paragraphs 47-48 regarding hybridization for twenty-one hours, which clearly qualifies as at least overnight, during the hybridization trial of Example III of the present application illustrates, to those of ordinary skill in the art of molecular biology, use of stringent hybridization conditions during the hybridization trial of Example III.

50. Next, we consider the salt concentration employed in the hybridization solution of Example III. Specifically, since the hybridization solution contained 0.8 M NaCl and 0.02 M pipes buffer (and assuming the pipes buffer employed NaOH), one of ordinary skill in the art of molecular biology would understand the hybridization solution contained about 0.82 M sodium ion. See Paragraph 45 above.

51. Those of ordinary skill in the art of microbiology know salt concentrations of about 0.82 M sodium ion are considered to be low salt concentrations generally sufficient to establish stringent conditions:

> Typically, stringent conditions will be those in which the salt concentration is less than about 1.0 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7. 0 to 8.3 . . . .

Page 12, lines 2-4 of Exhibit K.

52. The evidence provided in Paragraphs 45 and 50-51 regarding use of a hybridization solution with a salt concentration of about 0.82 M sodium ion during the hybridization trial of Example III of the present application illustrates, to those of ordinary skill in the art of molecular biology, use of stringent hybridization conditions during the hybridization trial of Example III. This statement is probative as to stringency even if the hybridization temperature of Example III is not taken into account. (See Paragraph 15 above).

53. Next, we consider the 50% formamide concentration employed in the hybridization solution of Example III. See Paragraph 45 above.

54. Beyond maintaining hybridization solution with relatively low ionic strength (i.e. a relatively low salt concentration), persons of ordinary skill in the art of molecular biology know another approach to attaining stringent hybridization conditions entails incorporation of formamide in the hybridization solution:

> Things that affect stringency:
> - Salt (NaCl) concentration (lower = high stringency)
> - Formamide concentration (higher = high stringency)
> - Temperature of incubation (higher = high stringency)

Slide 21 of a PowerPoint Presentation entitled Northern and Southern Blotting by Todd Lamitina, Research Fellow in the Department of Anesthesiology, Research Division, at Vanderbilt University Medical Center (Obtained on December 1, 2004 from the Internet at: http://bret.mc.vanderbilt.edu/igp/html/Methods_2003/Blotting.ppt) (attached as Exhibit N).

55. Persons of ordinary skill in the art of molecular biology know addition of formamide at a particular annealing temperature (T_M) enhances hybridization stringency by destabilizing the helical form of the nucleotides being hybridized: "*Formamide* is a helix destabiliser . . . ." Rybicki, Ed; Detection of Nucleic Acids by Hybridisation: Molecular Biology Techniques Manual (3rd ed. 1998) (attached as Exhibit O). By virtue of this helix destabilization, the formamide "'opens' nucleic acid." Slide 19 of Exhibit N.

56. Persons of ordinary skill in the art of molecular biology know that use of formamide at a concentration of 50% in the hybridization solution is generally sufficient to establish stringent conditions:

> After transfer, blots were hybridized at high stringency [50% formamide, 5X sodium chloride-sodium phosphate-EDTA (SSPE) (1X SSPE is 0.15 M NaCl, 10 mM NaH2PO4, and 1 mM EDTA), 5X Denhardt's solution, 0.2% SDS, and 10 μg/ml herring sperm DNA at 42°C] with PEPT1, PEPT2, SGLT1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA fragments labeled with [α-32P]dCTP as probes.

Page G58 of Xiaoyue Pan, Tomohiro Terada, Megumi Irie, Hideyuki Saito, And Ken-ichi Inui; Diurnal Rhythm of H^+-peptide Cotransporter in Rat Small Intestine; Am. J. Physiol. Gastrointest. Liver. Physiol. 283: G57–G64 (2002) (attached as Exhibit P). Further support for this exists:

> Membranes were hybridized either under conditions of moderate stringency [hybridization in 35% formamide, 0.1% Na-pyrophosphate, 50 mM Tris··Cl (pH 7.5), 5× SSC, 1% SDS, 5× Denhardt's, 100 μg/ml calf thymus DNA at 42°C, washing in 1× SSC/1% SDS at 60°C] or high stringency (hybridization in the same buffer except that the formamide concentration was 50% and at the same temperature . . . .

Page 11779 of Nanda, I., Kondo, M., Hornung, U., Asakawa, S., Winkler, C., Shimizu, A., Shan, Z., Haaf, T., Shimizu, N., Shima, A., Schmid, M., and Schartl, M., A Duplicated Copy of DMRT1 in the Sex-Determining Region of the Y Chromosome of the Medaka, *Oryzias Latipes*; Proc Natl Acad Sci USA; 2002 Sept. 3; 99(18): 11778-11783 (attached as Exhibit Q).

57. The evidence provided in Paragraphs 54-56 regarding use of a hybridization solution with a 50% concentration of formamide during the hybridization trial of Example III of the present application illustrates, to those of ordinary skill in the art of molecular biology, use of stringent hybridization conditions during the hybridization trial of Example III.

58. Building on the facts presented in Paragraph 54, persons of ordinary skill in the art of molecular biology know the addition of formamide allows the hybridization temperature to be decreased while maintaining the stringent hybridization conditions that would exist without changing the hybridization temperature and in the absence of the formamide:

> *Formamide* . . . enables one to decrease [the] annealing temperature: the presence of **formamide** decreases the Tm as shown:
>
> **TFm = Tm - 0.61(%formamide, w/v)**
>
> It is most often used in annealing reactions using RNA as target or probe, and especially with dsRNA hybrids, as these have high Tms which necessitate elevated reaction temperatures.

"Hybridization Stringency" section of Exhibit O.

59. Next, we consider the hybridization temperature employed in Example III. A pre-hybridization temperature of about 42°C is employed in Example III. See Paragraph 45 above. As evidenced below, those of ordinary skill in the art of molecular biology would therefore understand the hybridization temperature employed in Example III was also about 42°C.

60. Those of ordinary skill in the art of microbiology know the hybridization temperature is typically about the same as the prehybridization temperature that is employed in a particular hybridization trial:

> This step should always be carried out at the temperature of the hybridization.

"Prehybridization" section of Brochure entitled Southern (DNA) and Northern (RNA) Hybridizations by GE Osmonics, Inc. (2003) (Obtained on December 1, 2004 from the Internet at: http://www.msifilters.com/OsmoLabPage.dll?BuildPage&1&1&921) (attached as Exhibit R).

61. This knowledge by those of ordinary skill in the art of microbiology that the hybridization temperature is typically about the same as the prehybridization temperature that is employed in a particular hybridization trial is further exemplified by various sources: Compare numbered paragraphs 1 and 4 on the first page of Exhibit I, numbered paragraphs 1 and 4 on the second page of Exhibit I, and numbered paragraphs 1 and 4 on the third page of Exhibit I that each show the hybridization temperature and the prehybridization temperature were the same in any particular hybridization trial. As another example, see:

> The baked membranes were prehybridized using 25 mM potassium phosphate, 750 mM NaCl, 75 mM sodium citrate, 5XDenhardt's solution, 50 µg/ml denatured salmon sperm DNA and 50% formamide. After incubation for 14–16 h at 42°C, the membranes were hybridized with 32P-labeled probes in the prehybridization buffer plus 10% dextran sulfate. After hybridization for 14–16 h at 42°C . . . .

Page 351 of Scarpace, P. J., Nicolson, M., and Matheny, M.; UCP2, UCP3 and Leptin Gene Expression: Modulation by Food Restriction and Leptin Journal of Endocrinology 159, 349–357 (1998) (attached as Exhibit S).

62. A pre-hybridization temperature of about 42°C is employed in Example III. See Paragraph 45 above. Example III does not explicitly state that a hybridization temperature of about 42°C was employed in Example III. Nonetheless, the evidence presented in Paragraphs 59-61 above demonstrate those of ordinary skill in the art of molecular biology would understand the hybridization temperature employed in Example III was also about 42°C.

63. Continuing, we further consider the hybridization temperature of about 42°C those of ordinary skill in the art of molecular biology would understand was employed in Example III. See Paragraphs 45 and 62 above.

64. Those of ordinary skill in the art of molecular biology know use of a hybridization temperature of about 42 °C in combination with 50% formamide in the hybridization solution is generally sufficient to establish stringent conditions:

> Membranes were hybridized either under conditions of moderate stringency [hybridization in 35% formamide, 0.1% Na-pyrophosphate, 50 mM Tris··Cl (pH 7.5), 5× SSC, 1% SDS, 5× Denhardt's, 100 µg/ml calf thymus DNA at 42°C, washing in 1× SSC/1% SDS at 60°C] or high stringency (hybridization in the same buffer except that the formamide concentration was 50% and at the same temperature . . . .

Page 11779 of attached Exhibit Q. See also numbered para. 4 on the first page of Exhibit I, numbered para. 4 on the second page of Exhibit I, and numbered para. 4 on the third page of Exhibit I that each employ a hybridization temperature of about 42 °C in combination with 50% formamide, where such use of 50% formamide is understood by those of ordinary skill in the art of molecular biology as evidence stringent hybridization conditions were intended and employed. See page G58 of attached Exhibit P and page 11779 of attached Exhibit Q.

65. The evidence provided in Paragraphs 58-64 regarding use of a hybridization temperature of about 42 °C in combination with 50% formamide in the hybridization solution during the hybridization trial of Example III of the present application illustrates, to those of ordinary skill in the art of molecular biology, use of stringent hybridization conditions during the hybridization trial of Example III.

66. Next, we consider the salmon sperm concentration of 100 μg/ml that was employed in the hybridization solution of Example III. See Paragraph 45 above.

67. Persons of ordinary skill in the art of molecular biology know another technique for improving specificity of base pairing entails incorporation of non-specific DNA in the hybridization solution. See Exhibit E, as discussed in Paragraph 17 above. The non-specific DNA is said to prevent the probe from hybridizing nonspecifically - that is with nucleic acid

that is not complementary (specific) to the probe nucleic acid. See Exhibit F, as discussed at Paragraph 17 above.

68. Salmon sperm at a concentration of 100 μg/ml is incorporated by those skilled in the art of molecular biology to complement and support high stringency hybridization by limiting non-specific nucleic acid binding during hybridization:

> In a most preferred embodiment, a nucleic acid sequence that is hybridizable to a nucleic acid sequence (or a complement of the foregoing) encoding the aforementioned peptides, or a derivative of the same, under conditions of high stringency is provided. By way of example and not limitation, procedures using such conditions of high stringency are as follows: Step 1: Filters containing DNA are pretreated for 8 hours to overnight at 65°C in buffer composed of 6× SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500 µg/ml denatured salmon sperm DNA. Step 2: Filters are hybridized for 48 hours at 65°C in the above prehybridization mixture to which is added 100 mg/ml denatured salmon sperm DNA and 5-20××10^6 cpm of ^{32}P-labeled probe.

See column 14, lines 33-47, of Exhibit L.

69. The evidence provided in Paragraphs 66-68 regarding use of a hybridization solution with a salmon sperm concentration of 100 μg/ml during the hybridization trial of Example III of the present application illustrates, to those of ordinary skill in the art of molecular biology, use of conditions that inhibit non-specific probe hybridization and therefore further support attainment of results consistent with use of stringent conditions during the hybridization trial of Example III.

70. We next consider the post-hybridization washing conditions of Example III that concluded with a final wash containing "0.1 x SSC, 0.1% SDS" at 60°C for 30 minutes. See Paragraph 45 above.

71. As noted above, beyond controlling the hybridization stringency directly, by varying the temperature at which hybridization is performed and/or the chemical composition of the hybridization solution used during hybridization experiments , those of ordinary skill in the art of molecular biology know hybridization stringency may also be controlled by post-hybridization washing conditions, such as the temperature and/or the salt concentration. See Paragraph 19 above. For example:

> You can also control for stringency of hybridization at the next step, which is washing the unbound probe away from the blot. If you wash at high temperature or low salt concentration, you will remove any hybrids that are not perfectly matched.

See Exhibit H.

72. In this regard, those of ordinary skill in the art recognize high stringency washing may be accomplished using a temperature at least as high as the hybridization temperature in combination with the final washing solution of Example III that contained 0.1x SSC and 0.1% SDS:

> Following hybridisation, high stringency washing may be done in several steps, with a final wash (about 30 min) at the hybridisation temperature in 0.2-0.1 times SSC, 0.1% SDS.

See Paragraph 0188 of Exhibit M.

73. The evidence provided in Paragraph 72 demonstrates those of ordinary skill in the art of molecular biology recognize high stringency washing may be accomplished using a final washing solution containing 0.1x SSC and 0.1% SDS, as was employed in Example III. Recognizing that increased temperature generally increases stringency (see Paragraph 13 and Exhibit C, the evidence provided in Paragraph 72 further demonstrates those of ordinary skill in the art of molecular biology recognize high stringency washing may be accomplished at a temperature at least as high as the hybridization temperature; in Example III, the wash was

71. As noted above, beyond controlling the hybridization stringency directly, by varying the temperature at which hybridization is performed and/or the chemical composition of the hybridization solution used during hybridization experiments , those of ordinary skill in the art of molecular biology know hybridization stringency may also be controlled by post-hybridization washing conditions, such as the temperature and/or the salt concentration. See Paragraph 19 above. For example:

> You can also control for stringency of hybridization at the next step, which is washing the unbound probe away from the blot. If you wash at high temperature or low salt concentration, you will remove any hybrids that are not perfectly matched.

See Exhibit H.

72. In this regard, those of ordinary skill in the art recognize high stringency washing may be accomplished using a temperature at least as high as the hybridization temperature in combination with the final washing solution of Example III that contained 0.1x SSC and 0.1% SDS:

> Following hybridisation, high stringency washing may be done in several steps, with a final wash (about 30 min) at the hybridisation temperature in 0.2-0.1 times SSC, 0.1% SDS.

See Paragraph 0188 of Exhibit M.

73. The evidence provided in Paragraph 72 demonstrates those of ordinary skill in the art of molecular biology recognize high stringency washing may be accomplished using a final washing solution containing 0.1x SSC and 0.1% SDS, as was employed in Example III. Recognizing that increased temperature generally increases stringency (see Paragraph 13 and Exhibit C, the evidence provided in Paragraph 72 further demonstrates those of ordinary skill in the art of molecular biology recognize high stringency washing may be accomplished at a temperature at least as high as the hybridization temperature; in Example III, the wash was

accomplished at 60 °C, which is at least as high as the about 42 °C hybridization temperature employed in Example III. See Paragraphs 45 and 62.

74. The evidence presented in Paragraphs 44-73, as supplemented by the Exhibits referenced in Paragraphs 44-73 of my Declaration, illustrate that one of ordinary skill in the art, upon reviewing the hybridization conditions employed in Example III of the above-identified application, would recognize these hybridization conditions as being stringent hybridization conditions that would typically enable a probe, such as the bovine leptin cDNA molecule employed in Example III, to hybridize to a perfect or near perfect nucleic acid molecule complement of the probe, such as bovine leptin DNA.

75. As noted herein, the invention of the above-identified application is, in one aspect, directed to a variety of nucleic acid molecules (and functional variants thereof) that encode bovine adipocyte polypeptide leptin and are capable of hybridizing to nucleotide sequences (and portions thereof), and in another aspect is directed to hybridizing the nucleic acid molecules (and functional variants thereof) to nucleotide sequences (and portions thereof), such as various nucleotide sequences (and portions thereof) disclosed in the above-identified application. See Paragraphs 20-22 above.

76. The present application discloses conditions for hybridizing the nucleic acid molecules addressed in the present application (and in Paragraph 75) primarily in Examples II and III. As explained in Paragraphs 23, 44-45, and 74, one of ordinary skill in the art of microbiology, upon reviewing the hybridization conditions employed in Examples II and III of the above-identified application, would recognize these hybridization conditions as being stringent hybridization conditions that would typically enable a probe, such as the bovine leptin cDNA molecule employed in Examples II and III, to hybridize to a perfect or near

perfect nucleic acid molecule complement of the probe, such as bovine leptin mRNA or bovine leptin DNA.

77. Consequently, since the above-identified application primarily discloses to one of ordinary skill in the art of molecular biology use of stringent hybridization conditions for hybridizing the nucleic acid molecules addressed in the present application (and in Paragraph 75), it is evident that use of stringent hybridization conditions for hybridizing the nucleic acid molecules addressed in the present application is in fact disclosed and described in the present application.

78. We next consider the Examiner's comments in support of the Examiner's contention that U.S. Patent No. 6,309,853 to Friedman (subsequently referred to as "the Friedman patent") allegedly renders obvious certain aspects of the invention of the above-identified application directed to nucleic acids which encode porcine leptin and are capable of hybridizing to certain bovine leptin sequences (such as DNA and mRNA):

> The instant claims are directed to isolated nucleic acids which encode bovine leptin and hybridize SEQ ID NO:3 or a 'functional derivative thereof' (see claims 22, 27) or "variant"(see claims 24-26). The prior art of Friedman et al. (U.S. Pat. No. 6,309,853) disclose nucleic acids which encode human and mouse leptin, which would be considered functional derivatives and/or variants of SEQ ID NO:3 since they encode leptin molecules and would possess similar functional properties as those of the bovine leptin, absent evidence to the contrary. Friedman et al. teach that the leptin gene (or OB) could be isolated from domestic animals using the methods disclosed therein (see column 26, line 53 to column 27, line 49). Friedman et al. specifically mention cattle as a domestic animal for which leptin would be useful (see column 48, lines 41-57). Friedman et al. do not specifically disclose an isolated nucleic acid encoding a bovine leptin polypeptide. However, it would have been obvious to use the nucleic acid of Friedman et al. encoding human or mouse leptin and hybridize it to a bovine cDNA library and isolate a nucleic acid molecule encoding bovine leptin because Friedman et al. teach methods for isolating leptin encoding nucleic acids and also teach that it would be beneficial to administer leptin to cattle. Therefore, the invention

> as a whole would have been obvious at the time it was made, absent evidence to the contrary.

Office Action dated June 16, 2004 in the above-identified application.

79. I am experienced in isolating, purifying, and manipulating DNA sequences, such as bovine, porcine, murine, and human DNA sequences, such as leptin DNA sequences, due to my education and work experience relating to DNA sequence and leptin genetics issues over the past twenty-five years. See Paragraphs 1-9 above.

80. I am familiar with and have carefully and thoroughly reviewed the Friedman patent which the Examiner relies upon in the Examiner's statement recited in Paragraph 78.

81. Based on my thorough review of the Friedman patent, I note, consistent with the Examiner's observation recited in Paragraph 78, the Friedman patent discloses murine and human leptin DNA sequences and polypeptides.

82. Based on my careful review of the Friedman patent and consistent with the Examiner's observation recited in Paragraph 78, the Friedman patent does not disclose any bovine leptin DNA (or mRNA) molecules or polypeptides.

83. Based on my thorough review of the Friedman patent and consistent with the Examiner's observation recited in Paragraph 78, the Friedman patent does not disclose any functional derivative or variant DNA (or mRNA) molecules that encode for bovine leptin polypeptide.

84. Based on my careful review and comparison of the human and murine leptin DNA sequences disclosed in the Friedman patent and the bovine leptin DNA sequences disclosed in the above-identified application, it is my observation that the human and murine leptin DNA

sequences disclosed in the Friedman patent differ substantially from the bovine leptin DNA sequences disclosed in the above-identified application; consequently, the bovine leptin of the present application that is based on the bovine leptin DNA sequences (and functional variants thereof) disclosed in the above-identified application differs in substantial detail from both the human leptin and the murine leptin disclosed in the Friedman patent.

85. As a result, based on my observation in Paragraph 84, the bovine leptin of the present application is functionally, different from both the human leptin and the murine leptin disclosed in the Friedman patent, as discussed more fully in subsequent Paragraphs of my Declaration.

86. For example, when recombinant growth hormone is administered by injection to castrate male cattle, the castrate male cattle exhibit increased adipose tissue leptin mRNA expression. Houseknecht, K. L.; Portocarrero, C. P.; Ji, S.; Lemenager, R.; and Spurlock, M. E.; Growth Hormone Regulates Leptin Gene Expression in Bovine Adipose Tissue: Correlation With Adpose IGF-1 Expression, Journal of Endocrinology, Vol 164, pages 51-57 (2000) (attached as Exhibit T).

87. On the other hand, when recombinant growth hormone is administered by injection to male mice, the male mice exhibit essentially no increased adipose tissue leptin mRNA expression. Lee, K. N.; Jeong, I.C.; Lee, S. J.; Oh, S. H.; and Cho, M. Y.; Regulation of Leptin Gene Expression by Insulin and Growth Hormone in Mouse Adipocytes, Experimental and Molecular Medicine, Vol. 33, No. 4, pages 234-239 (2001) (attached as Exhibit U).

88. Since recombinant growth hormone administration increases adipose tissue leptin mRNA expression in male cattle, while recombinant growth hormone administration causes essentially no increased adipose tissue leptin mRNA expression in male mice, the effects of

growth hormone administration on leptin mRNA expression in male cattle versus in male mice differ dramatically, and it is consequently evident the bovine leptin protein is functionally very different from the rat leptin protein.

89. I have carefully reviewed and am familiar with the publication of Exhibit T by Houseknecht, K. L.; Portocarrero, C. P.; Ji, S.; Lemenager, R.; and Spurlock, M. E.; Growth Hormone Regulates Leptin Gene Expression in Bovine Adipose Tissue: Correlation With Adpose IGF-1 Expression, Journal of Endocrinology, Vol 164, pages 51-57 (2000) (subsequently referred to as "the Spurlock publication") that documents the increased adipose tissue leptin mRNA expression in cattle after recombinant bovine growth hormone administration.

90. In rodents and humans, leptin gene expression is under complex endocrine and metabolic control and is strongly influenced by energy balance. Leptin regulates food intake and whole-body energy metabolism. Similarly, growth hormone (GH) plays in important role in the regulation of whole-body energy utilization. GH treatment attenuates insulin-stimulated lipogenesis and enhances the adipocyte response to adrenergic stimulation of lipolysis, with the net effect of partitioning nutrients away from the fat cell. (See the Introduction section on pages 51-52 of the Spurlock publication of Exhibit T).

91. Therefore, a primary aim of the study documented in the Spurlock publication was to determine the effect of short-term GH treatment on healthy, growing cows in terms of leptin gene expression in subcutaneous adipose tissue, before the occurrence of GH-induced changes in adiposity. (See the Introduction section on pages 51-52 of the Spurlock publication of Exhibit T).

92. According to the Spurlock publication, twelve castrate Angus male cows weighing about 296.9 ± 27 kg were fed, *ad libitum*, a standard complete diet for growing cows. The diet was

formulated from corn, corn silage and protein-mineral supplement to meet National Research Council requirements for growing cattle. (See Materials and Method section on page 52 of the Spurlock publication of Exhibit T).

93. With each cow serving as its own control, cows were randomly assigned to the following groups:

Group 1: Control group receiving saline injections (200 µg per kg body weight per day) for 3 days.

Group 2: Treated group receiving GH injections (200 mg per kg body weight per day) for 3 days.

(See Materials and Method section on page 52 of the Spurlock publication of Exhibit T).

94. The concentration of hormones administered to the male cows were within the ranges reported to be effective in regulating leptin expression in rodent and human adipose tissue. (See Materials and Method section on page 52 of the Spurlock publication of Exhibit T).

95. Approximately eighteen hours after the third injection of saline or GH, a subcutaneous adipose tissue sample was obtained by surgical biopsy from each animal. (See Materials and Method section on page 52 of the Spurlock publication of Exhibit T).

96. After a 7-day interim period for recovery from the previous experimental treatment and biopsy surgery documented in Paragraphs 94-95, the cows received the alternate treatment (cows previously receiving the Group 1 treatment now received the Group 2 treatment, and cows previously receiving the Group 2 treatment now received the Group 1 treatment); a second subcutaneous adipose tissue biopsy was obtained from each cow approximately eighteen hours after the third injection of saline or GH. All adipose tissue samples were snap frozen after being obtained and stored at -80°C until required for analysis for leptin mRNA abundance. (See Materials and Method section on page 52 of the Spurlock publication of Exhibit T).

97. Total RNA was extracted from tissue and analyzed for bovine leptin mRNA by ribonuclease protection analysis using a bovine-specific leptin ribonucleotide probe (riboprobe). The following steps were used to quantify the abundance of bovine leptin mRNA in subcutaneous adipose tissue:

A. First, total RNA was extracted from adipose tissue using an acidic guanidinium thiocyanate-phenol-chloroform extraction technique based on the method of Chomczynski and Saachi (Chomczynski & Sacchi, 1987, Analytic Biochemistry 162:156).

B. Next, the bovine-specific leptin riboprobe was prepared by synthetically adding a 27-bp bacteriophage T7 promoter sequence to a 5' end of antisense leptin primer as described by Ji et al. (Ji et al., Animal Biotechnology 9(1) 1-14) to form the bovine-specific riboprobe.

C. Approximately 50 μCi (α-^{32}P] UTP (800 Ci/mmol) was used during *in vitro* transcription of the bovine-specific riboprobe with T7 RNA polymerase. After transcription, a radioactively labeled bovine-specific riboprobe was obtained.

D. Ten micrograms of bovine adipose RNA obtained in Paragraph No. 97A was hybridized to the radioactively labeled bovine specific riboprobe overnight at 45 °C. For quantification purposes, the bovine adipose RNA was also co-hybridized to an 18S probe (Ambion).

E. After hybridization, any single-stranded RNA molecules were digested with a 1:50 mixture of RNase I (250 U/ml)-RNase T1 (10 000 U/ml) for one hour and protected bands (RNA probe + RNA target) were separated by gel electrophoresis (8 M urea, 5% acrylamide).

F. Separated band intensities were quantified by densitometry or cutting the gel and quantifying radioactivity in gel slices.

98. According to the Spurlock publication, growth hormone treatment significantly increased leptin mRNA abundance in cows exhibiting a positive IGF-1 response. (See the Results and Discussion section on page 54 of the Spurlock publication of Exhibit T). A positive IGF-1 response indicates a normal response of cows to recombinant growth hormone administration. (See the Results and Discussion section on page 52 of the Spurlock publication of Exhibit T).

99. This increased adipose tissue leptin mRNA abundance mentioned in Paragraph 98 after recombinant bovine growth hormone administration in male cattle is in contrast to the effects observed after recombinant growth hormone administration to male mice, where recombinant growth hormone administration did not have an effect on mRNA transcription of the leptin gene. See Paragraphs 87 and 88 above.

100. I have carefully reviewed and am familiar with the publication of Exhibit U by Lee, K. N.; Jeong, I.C.; Lee, S. J.; Oh, S. H.; and Cho, M. Y.; Regulation of Leptin Gene Expression by Insulin and Growth Hormone in Mouse Adipocytes, Experimental and Molecular Medicine, Vol. 33, No. 4, pages 234-239 (2001) (subsequently referred to as "the Lee publication") that documents the effect of recombinant growth hormone administration in male mice to causes essentially no increase of adipose tissue leptin mRNA expression in the male mice.

101. According to the Lee publication, fifteen male ICR mice weighing about 50 grams were fed commercial rat chow *ad libitum*. (See the Materials and Methods section on page 235 of the Lee publication of Exhibit U).

102. The male mice were divided into five groups (three mice per group), and 0.6 units of GH (dissolved in physiological saline) per mouse per day were injected subcutaneously (sc). Like the male cows of the Spurlock publication, GH injections were administered to the mice

for three days. The control group of mice received the same amount of physiological saline solution for three days as well. (See the Materials and Methods section on page 235 of the Lee publication of Exhibit U).

103. All mice were sacrificed after blood was drawn from the mice. Adipose tissue in epididymal fat was removed and washed twice with saline solution and saved for analysis of leptin expression. (See the Materials and Methods section on page 235 of the Lee publication of Exhibit U).

104. Total RNA in mouse adipose tissue was isolated using Tri agent (Amersham Life Science Ltd., Buckinghamshire, England) according to the method described in the manual supplied by Amersham Life Science Ltd. (See the Materials and Methods section on page 235 of the Lee publication of Exhibit U).

105. Reverse transcription of leptin mRNA was carried out by incubating a mixture (final 20μl) containing 1 μg of total RNA dissolved in 8 μl of DEPC-treated water with 2 μl of 5 x reaction buffer (250 mM Tris-Cl, pH 8.3, 25 mM KCl, 50 mM DTT, 50 mM $MgCl_2$) at 65°C for 10 minutes, followed by incubation for one hour at 37°C after addition of 1 μl of antisense primer for leptin cDNA (100 pmol), 1 ml of dNTP (10 mM), 1 ml of RNase inhibitor (20 units), and 1 μl of reverse transcriptase (10 units). (See the Materials and Methods section on page 235 of the Lee publication of Exhibit U).

106. After the incubation, the reaction mixture was placed in boiling water and incubated for five minutes to inactivate the enzyme. Quantitative PCR was performed by the method of Saiki *et al.* (1988) using sense and antisense primers corresponding to leptin cDNA sequence together with leptin cDNA as template. (See the Materials and Methods section on page 235 of the Lee publication of Exhibit U).

107. Total RNAs isolated from adipose tissue of mice administered with various hormones were subjected to formaldehyde agarose gel electrophoresis according to the previously described method (Kim *et al.*, 2000). (See the Materials and Methods section on page 235 of the Lee publication of Exhibit U).

108. RNAs separated on the gel were electrophoretically transferred onto nitrocellulose membrane. Leptin mRNA was hybridized with ^{32}P-labeled leptin cDNA and detected by autoradiography. (See the Materials and Methods section on page 235 of the Lee publication of Exhibit U).

109. According to the Lee publication, subcutaneous administration of growth hormone to male mice fed *ad libitum* had no effect on mRNA transcription of leptin gene:

> The level of leptin mRNA in adipose tissue was increased by insulin (0.6 units/mouse) administration, but the administration of growth hormone (0.3 or 0.6 units/mouse) had no effect on mRNA transcription of leptin gene with (Figure 5).

(See the Results section on page 237 and Figure 5 of the Lee publication of Exhibit U).

110. Therefore, the authors of the Lee publication concluded growth hormone administration to male mice fed *ad libitum* had essentially no (little effect) on leptin mRNA transcription in mouse adipose tissue:

> In the present study, administration of insulin to mice increased leptin mRNA transcription in adipose tissue, while growth hormone caused a marginal increase. Consequently, the stimulatory effect of insulin on leptin mRNA transcription was obvious *in vivo* as well as in cell culture system, however, growth hormone had little effect on leptin mRNA transcription in mouse adipose tissue.

(See the Discussion section on page 238 of the Lee publication of Exhibit U).

111. The observation of essentially no effect in adipose tissue leptin mRNA expression by the male mice (see Paragraphs 109-110) by the authors of the Lee publication contrasts sharply to the observed increase in adipose tissue leptin mRNA expression after administration of recombinant bovine growth hormone to male cattle, as detailed in the Spurlock publication. See Paragraphs 98-99 and 109-110.

112. Therefore, based on the documented differences in adipose tissue leptin mRNA expression after administration to normal male cows (per the Spurlock publication) versus normal male rats (per the Lee publication), it is evident that bovine leptin surprisingly functions very differently after administration of growth hormone to male cattle as compared to how rat leptin functions after administration of growth hormone to male rats. See Paragraphs 98-99 and 109-110.

113. As another example, when dexamethasone (a glucocorticoid) is administered to multiparous non-lactating Holstein cows, the dexamethasone administration fails to change plasma leptin protein levels. Maciel, S. M.; Chamberlain, C. S.; Wettermann, R. P.; and Spicer, L. J.; <u>Dexamethasone Influences Endocrine and Ovarian Function in Dairy Cattle,</u> Journal of Dairy Science 84:1998-2009 (2001) (attached as Exhibit V).

114. On the other hand, when dexamethasone is administered to healthy human volunteers, the healthy human volunteers exhibit <u>significantly</u> <u>increased</u> leptin expression in the serum after dexamethasone administration. Papaspyrou-Rao, S.; Schneider, S. H.; Peterson, R.N.; and Fried, S. K.; <u>Dexamethasone Increases Leptin Expression in Humans *In Vivo*</u>, Journal of Clinical Endocrinology and Metabolism, Vol 82, No. 6, pages 1635-1637 (1997) (attached as Exhibit W).

115. Since dexamethasone administration <u>fails</u> <u>to</u> <u>change</u> plasma leptin concentrations in cows, while dexamethasone administration significantly <u>increased</u> serum leptin levels in healthy human volunteers, it is evident bovine leptin functions very different from human leptin.

116. I have carefully reviewed and am familiar with the publication of Exhibit V by Maciel, S. M.; Chamberlain, C. S.; Wettermann, R. P.; and Spicer, L. J.; entitled Dexamethasone Influences Endocrine and Ovarian Function in Dairy Cattle, Journal of Dairy Science 84:1998-2009 (2001) (subsequently referred to as "the Maciel publication") that documents the unchanged plasma leptin concentration after dexamethasone administration.

117. In the study documented in the Maciel publication, eleven non-lactating multiparous Holstein cows were divided into two groups: dexamethasone-treated (n=6) and control (n=5) cows. Both groups of cows were synchronized with $PGF_{2\alpha}$ (a hormone) using two injections 25 mg each (intramuscularly) spaced eleven days apart. (See the Materials and Methods section on page 1999 of the Maciel publication of Exhibit V).

118. After synchronization, and thereafter starting one day after ovulation, the cows of the treatment group received daily intramuscular injections of 44 μg dexamethasone per kilogram of body weight. The cows of the control group received daily intramuscular injections of one milliliter of a vehicle solution (containing ethanol, polyethylene glycol, and sterile filtered Milipore water at a pH of 4.9) per 100 kg of body weight. (See the Materials and Methods section on page 1999 of the Maciel publication of Exhibit V).

119. All cows of both treatment groups were managed as one herd and housed in a dry lot with access to shelter and water. During the experiment, daily feeding consisted of two kilograms of grain and seven kilograms of sorghum silage per head, as fed, and free choice of bermuda grass hay, containing 16%, 8% and 13% (% crude protein, dry matter basis), respectively). (See the Materials and Methods section on page 1999 of the Maciel publication of Exhibit V).

120. According to the Maciel publication, dexamethasone treatment had no effect on plasma leptin concentrations in the cows. Plasma leptin concentrations averaged 7.78 ± 1.16 and 7.25 ± 1.27

ng/ml in the dexamethasone-treated cows and the controls cows, respectively. (See the Results section on page 2004 of the Maciel publication of Exhibit V).

121. This non-effect on plasma leptin concentration that is noted in Paragraph 120 after dexamethasone administration in cows is in contrast to the effects observed after dexamethasone administration to healthy human volunteers, where dexamethasone administration significantly increased serum leptin levels. See Paragraph 114 above.

122. I have carefully reviewed and am familiar with the publication of Exhibit W by Papaspyrou-Rao, S.; Schneider, S. H.; Peterson, R.N.; and Fried, S. K.; Dexamethasone Increases Leptin Expression in Humans *In Vivo*, Journal of Clinical Endocrinology and Metabolism, Vol 82, No. 6, pages 1635-1637 (1997) (subsequently referred to as "the Fried publication") that documents the increased serum leptin levels in healthy human volunteers after dexamethasone administration.

123. According to the Fried publication, eight healthy subjects with a body mass index (BMI) of about 26.7 kg/m^2 ± 1.5 and an age of 31 ± 2 years were recruited for Study 2 and subjected to serum sampling after a twelve hour overnight fast. Serum insulin, cortisol, glucose and leptin were measured. (See Materials & Methods section on page 1635 of the Fried publication of Exhibit W).

124. After serum sampling, healthy human volunteers were instructed to ingest tablets containing a total of 1.5 mg dexamethasone per day (0.75 mg, 2 x daily with breakfast and dinner) on two consecutive days. (See Materials and Methods section on page 1635 of the Fried publication of Exhibit W).

125. After an overnight fast following dexamethasone administration, the volunteers were subjected to a second blood sample to determine serum insulin, cortisol, glucose and leptin concentrations. (See Materials and Methods section on page 1635 of the Fried publication of Exhibit W).

126. Leptin was quantified using a radioimmunoassay kit from Linco of St. Charles, Missouri. (See Materials and Methods section on page 1635 of the Fried publication of Exhibit W).

127. According to the Fried publication, ingestion of dexamethasone by the human volunteers significantly increased serum leptin levels:

> In study 2, serum leptin levels increased by 80 ± 17%, n = 9, after two days of dexamethasone (P < 0.005; Fig 2).

(See the Results section on page 1636 of the Lee publication of Exhibit W).

128. Therefore, the authors of the Fried publication concluded that dexamethasone administration to healthy human volunteers increased serum leptin levels by 80%:

> We have demonstrated that induction of mild hypercortisolemia for two days increased leptin mRNA levels by 70% in both gluteal and abdominal subcutaneous adipose tissues and increased plasma leptin levels by 80%.

(See the Discussion section on page 1636 of the Fried publication of Exhibit W).

129. Consequently, based on the documented differences in bovine leptin protein expression after dexamethasone administration to normal cows (per the Spurlock publication) versus human leptin protein expression after dexamethasone administration to healthy human volunteers (per the Fried publication), it is evident that bovine leptin protein surprisingly functions very differently from human leptin protein. See Paragraphs 113-115, 120-121 and 127-128.

130. The foregoing factual evidence illustrates the human leptin disclosed in the Friedman patent does not, despite the Examiner's contentions to the contrary (see Paragraph 78), necessarily, or actually, possess functional properties similar to the functional properties of the porcine leptin disclosed in the above-identified application.

131. For example, based on the documented differences in adipose tissue leptin mRNA expression after growth hormone administration to male cows (per the Spurlock publication) versus adipose tissue leptin mRNA expression after growth hormone administration to male rats (per the Lee publication), it is evident bovine leptin protein surprisingly functions very differently as compared to how rat leptin protein functions. See Paragraphs 86-88, 98-99, and 109-111.

132. The documented differences in leptin mRNA expression after growth hormone administration to normal cows (per the Spurlock publication) versus normal rats (per the Lee publication) are surprising and unexpected, since there is no evidence of record, such as in the Spurlock publication, the Lee publication, or the Friedman patent, that would suggest the differential effects on leptin mRNA expression caused by growth hormone administration in male cows versus growth hormone administration in male rats.

133. Furthermore, the documented differences in leptin mRNA expression after growth hormone administration to normal cows (per the Spurlock publication) versus growth hormone administration to normal rats (per the Lee publication) are surprising and unexpected, since there is no evidence of record, such as in the Spurlock publication, the Lee publication, or the Friedman patent, that would suggest growth hormone administration in rats would have essentially no effect on leptin mRNA expression by the rats, while growth hormone administration in cows would cause an <u>increase</u> in leptin mRNA expression by the cows.

134. Therefore, based on the factual results noted in Paragraphs 130-133 and despite the Examiner's contentions to the contrary (see Paragraph 78), the Examiner's speculative suggested hybridization of the nucleic acid of the Friedman patent that encodes murine leptin to a bovine DNA library and subsequent isolation of a nucleic acid molecule encoding bovine leptin is not suggested since the functional characteristics of the murine leptin disclosed in the Friedman patent would not confirm isolation of a nucleic acid molecule encoding for bovine leptin as claimed in the above-identified application.

135. Likewise, the foregoing factual evidence illustrates the human leptin disclosed in the Friedman patent does not, despite the Examiner's contentions to the contrary (see Paragraph 78), necessarily, or actually, possess functional properties that are similar to the functional properties of the bovine leptin disclosed in the above-identified application.

136. For example, based on the documented differences in plasma leptin concentrations after dexamethasone administration to normal cows (per the Maciel publication) versus plasma leptin concentrations after dexamethasone administration to healthy human volunteers (per the Fried publication), it is evident recombinant bovine leptin protein surprisingly functions very differently from human leptin protein. See Paragraphs 113-115, 120-121, and 127-129.

137. The documented differences in plasma leptin concentrations after dexamethasone administration to normal cows (per the Maciel publication) versus serum leptin levels after dexamethasone administration to healthy human volunteers (per the Fried publication) are surprising and unexpected, since there is no evidence of record, such as in the Maciel publication, the Fried publication, or the Friedman patent, that would suggest the differential effects on leptin secretion caused by dexamethasone administration to cows versus dexamethasone administration to humans.

138. Furthermore, the documented differences in plasma leptin concentrations after dexamethasone administration in healthy cows (per the Maciel publication) versus healthy human volunteers (per the Fried publication) are surprising and unexpected, since there is no evidence of record, such as in the Maciel publication, the Fried publication, or the Friedman patent, that would suggest dexamethasone administration to cows would not effect plasma leptin concentrations in the cows, while dexamethasone administration to humans would increase plasma leptin levels in humans.

139. Finally, despite the Examiner's contentions to the contrary (see Paragraph 78), the Examiner's speculative suggested hybridization of the nucleic acid of the Friedman patent that encodes human leptin to a bovine DNA library and subsequent isolation of a nucleic acid molecule encoding bovine leptin is not suggested since the functional characteristics of the human leptin disclosed in the Friedman patent would not necessarily confirm isolation of a nucleic acid molecule encoding for bovine leptin as claimed in the above-identified application.

140. I declare that all statements made herein that are of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

Name: Michael E. Spurlock

Signature [signature]

Date: Dec. 9, 2004

First Named Inventor	:	Michael E. Spurlock	
Appln. No.	:	09/928,522	
Filed	:	August 13, 2001	Group Art Unit: 1647
Title	:	Bovine Leptin Protein, Antisense and Antibody	Examiner: C. J. Saoud
Docket No.	:	LL31.12-0015	

EXHIBIT B

of

DECLARATION
UNDER
37 C.F.R. 1.131

SEQUENCE LISTING OF 450 BASE CLONE
THAT ACCOMPANIED THE LETTER OF
EXHIBIT A OF BRIAN HOFFMAN
OF NATIONAL BIOSCIENCES, INC.
TO DR. JI

DNASIS
JI CON

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JI R       10         20         30         40         50         60
ACCGCTGCAT TCAGGGCTGA GGTCCAGCTG CCGCAACATG TCCTGTAGTG ACCCCTGCAG
TGGCGACGTA AGTCCCGACT CCAGGTCGAC GGCGTTGTAC AGGACATCAC TGGGGACGTC

        70         80         90        100        110        120
CCGGCTCAGG GCCACCACCT CGGTGGAGTA GAGGGAGGCT TCCAGGACGA CACCCAAGCT
GGCCGAGTCC CGGTGGTGGA GCCACCTCAT CTCCCTCCGA AGGTCCTGCT GTGGGTTCGA

       130        140        150        160        170        180
CTCCAAGCTC TCCAGGGCCC TGAACTGCGG CAAGGGGCAG CTCTTGGAGG CGGCCAGCAG
GAGGTTCGAG AGGTCCCGGG ACTTGACGCC GTTCCCCGTC GAGAACCTCC GCCGGTCGTC
                           ← JI.F

       190        200        210        220        230        240
GTGGAGAAGG TCCCGGAGGT TCTCCAGGTC ATTGGATATT TGGACCACAT TTCTGGAAGG
CACCTCTTCC AGGGCCTCCA AGAGGTCCAG TAACCTATAA ACCTGGTGTA AAGACCTTCC

       250        260        270        280        290        300
CAGACTGGTG AGGATCTGTT GGTAGATCGC CAATGTCTGG TCCATCTTGG ACAAACTCAG
GTCTGACCAC TCCTAGACAA CCATCTAGCG GTTACAGACC AGGTAGAACC TGTTTGAGTC

       310 JI R →     320        330        340        350        360
GAGAGGGTGG AGCCCAGGGA TGAAGTCCAA ACCAGTGACC CTCTGTTTGG AGGAGACGGA
CTCTCCCACC TCGGGTCCCT ACTTCAGGTT TGGTCACTGG GAGACAAACC TCCTCTGCCT

       370        380        390        400        410        420
CTGCGTGTGT GAGATGTCAT TGATCCTGGT GACAATTGTC TTGATGAGGG TTTTGGTGTC
GACGCACACA CTCTACAGTA ACTAGGACCA CTGTTAACAG AACTACTCCC AAAACCACAG

       430        440        450        460        470        480
ATCCTGGACT TTTTGGATAG GCACGGCCT. .......... .......... ..........
TAGGACCTGA AAAACCTATC CGTGCCGGA. .......... .......... ..........
                        JI F
```

First Named Inventor	:	Michael E. Spurlock	
Appln. No.	:	09/928,522	
Filed	:	August 13, 2001	Group Art Unit: 1647
Title	:	Bovine Leptin Protein, Antisense and Antibody	Examiner: C. J. Saoud
Docket No.	:	LL31.12-0015	

EXHIBIT A

OF

AMENDMENT AFTER FINAL

Y.M. Kennes, B.D. Murphy, F. Pothier and M.-F Palin, entitled

Characterization of Swine *Leptin* (*Lep*) Polymorphisms

and Their Association with Production Traits (2001)

SHORT COMMUNICATION

Characterization of swine *leptin* (*LEP*) polymorphisms and their association with production traits

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Summary

Four polymorphisms in the swine *leptin* (*LEP*) gene were characterized and evaluated for association with economically important production traits in Yorkshire, Landrace and Duroc pigs. Our results show that these polymorphisms are generally of low frequency or are absent in pig populations. Two polymorphisms (A2845T and T3469C) may be associated ($P < 0.0078$) with feed intake and growth rate traits in Landrace pigs.

Keywords *LEP* gene, leptin, pig, polymorphism.

Leptin, the product of the *LEP* gene, is secreted mainly by adipose tissue and acts as a satiety signal on the hypothalamus, thereby regulating body weight and energy expenditure (Campfield *et al.* 1995). In swine, leptin mRNA levels are greater in adipose tissue from obese pigs than lean pigs (Robert *et al.* 1998; McNeel *et al.* 2000). Furthermore, injection of recombinant porcine leptin reduces feed intake and increases growth hormone (GH) secretion in swine (Barb *et al.* 1998). These observations suggest that *LEP* may be a candidate gene for economically important production traits such as backfat thickness, feed intake and growth rate in swine. The objectives of the present study were to estimate the frequency of previously reported DNA polymorphisms (Stratil *et al.* 1997; Robert *et al.* 1998) in different porcine breeds, and to investigate their association with production traits.

The polymerase chain reaction (PCR) amplifications of three genomic fragments of the porcine *LEP* gene that contain polymorphisms were performed. Primers used in the study were based on available genomic (Bidwell *et al.* 1997; GenBank SSU66254) and mRNA sequences (Robert *et al.* 1998; GenBank AF026976). The first primer pair (foward 5′-AGAGTCCAGGATGACAC-3′; reverse 5′-ATCTGTTGGT-AGATCGC-3′) amplified a region from nucleotide 976–3562 (Bidwell *et al.* 1997). The PCR reaction was carried out in a 50-µl total volume containing 500 ng of genomic DNA, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 2.6 units of Expand HF PCR System enzyme mix (Roche Molecular Biochemicals, Mannheim, Germany), 1X Expand HF buffer and 0.3 µM of each primer. The PCR profile was 2 min at 95 °C; 10 cycles of 15 s at 95 °C, 30 s at 50 °C, 2 min at 68 °C; followed by 37 cycles of 15 s at 95 °C, 30 s at 52 °C, 2 min at 68 °C with an increment of 5 s for each cycle, and a final 7 min extension at 68 °C. This PCR amplification generated a fragment of 2586 bp (fragment 1).

Primers for fragment 2 (foward 5′-AGCAGTCTGTCTCC-TCCAAAC-3′; reverse 5′-AGTAATAGATGCTGATGCGCC-3′) amplified a region from nucleotides 3398 to 5480 (2082 bp total, Bidwell *et al.* 1997), whilst fragment 3 primers (foward 5′-CCCTGCTTGCACTTGGTAGC-3′; reverse 5′-CTGCCACA-CGAGTCTTGCTC-3′) amplified a region from nucleotides 2237 to 2895 (658 bp total, Robert *et al.* 1998). The PCR reactions of both fragments 2 and 3 were performed in a 100-µl total volume containing 50 ng of genomic DNA, 0.2 mM of each dNTP, 1.0 mM $MgCl_2$, 2.5 units of *Taq* DNA polymerase, 1X *Taq* polymerase buffer and 0.4 µM of each primer. The PCR profile was 2 min at 94 °C, 38 cycles of 1 min at 94 °C, 1 min at 64 °C (fragment 2) or 66 °C (fragment 3), 1 min at 72 °C, and a final 5 min extension at 72 °C.

Amplified fragments were digested with *Xba*I (fragment 1), *Bgl*II and *Hin*fI (fragment 2), or *Hin*dIII (fragment 3). Figure 1 illustrates the fragment patterns of the *Xba*I, *Bgl*II and *Hin*dIII DNA polymorphisms. Fragments corresponding to each allele of the *Xba*I, *Bgl*II and *Hin*dIII DNA polymorphisms were sequenced on an ABI PRISM 377

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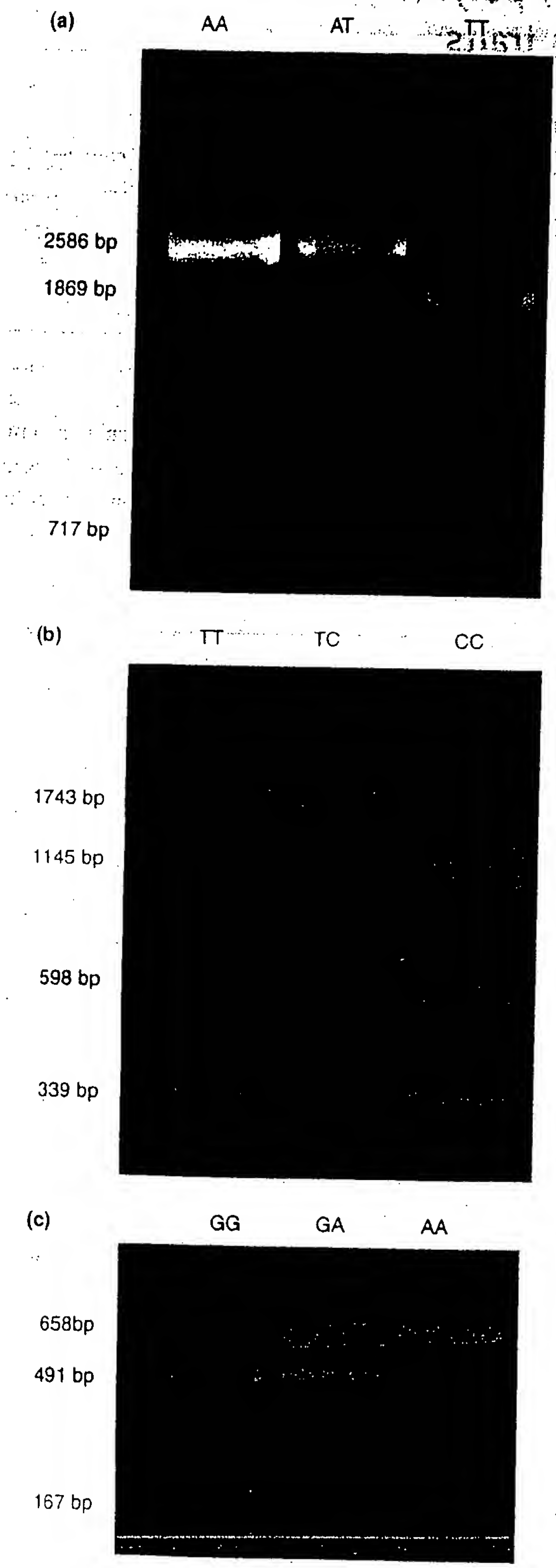

Figure 1 (a) Observed genotypes of the porcine *LEP* A2845T polymorphism after *Xba*I digestion of fragment 1: AA homozygote, 2586 bp; AT heterozygote, 2586 + 1869 + 717 bp; TT homozygote, 1869 + 717 bp. (b) Observed genotypes of the porcine *LEP* T3996C polymorphism after *Bgl*II digestion of fragment 2: TT homozygote, 1743 + 339 bp; TC heterozygote, 1743 + 1145 + 598 + 339 bp; CC homozygote, 1145 + 598 + 339 bp. (c) Observed genotypes of the porcine *LEP* G2728A polymorphism after *Hin*dIII digestion of fragment 3: GG homozygote, 491 + 167 bp; GA heterozygote, 658 + 491 + 167 bp; AA homozygote, 658 bp. Genotypes of individuals are indicated above the lanes.

automated DNA sequencer revealing A2845T, T3996C and G2728A polymorphisms, respectively. The A2845T substitution occurred in the second intron of the *LEP* gene whilst T3996C and G2728A substitutions were in the 3′ untranslated region (3′ UTR) region. Analyses also confirmed the *Hin*fI polymorphism identified by Stratil *et al.* (1997) and characterized by a T3469C substitution in the second exon.

Genotypes were assayed in two sample groups from the 'Programme d'Évaluation des Porcs en Station' population, kindly provided by the Centre de Développement du Porc du Québec. In the first sample, pigs were selected according to the highest and lowest estimated breeding values (EBV) for age (days; adjusted to 100 kg) and backfat thickness (mm; adjusted to 100 kg) (Kemp & Rothschild 1994), generating two subgroups (positive and negative). Backfat thickness was measured between the third and fourth last ribs, 5 cm off the mid-line of the split carcass. The positive subgroup included animals with lower genetic growth potential (more days to reach 100 kg) and higher backfat thickness values at 100 kg. The negative subgroup included animals with higher genetic growth potential and lower backfat thickness at 100 kg. Age- and fat-EBV values of 0 corresponded to the average of genetic growth potential and genetic leanness potential for all contemporary purebred pigs assessed in Canada. The positive subgroup included pigs with positive age- and backfat-EBV (age-EBV ≥ 0.9; backfat-EBV ≥ 0.2) whilst the negative subgroup included pigs with negative age- and backfat-EBV (age-EBV ≤ -1.6; backfat-EBV ≤ -0.3). In total, the first sample included 182 unrelated pigs separated by two generations, including 46 Duroc, 58 Landrace and 78 Yorkshire. This pig sample was analysed using Fisher's exact test (two-tailed).

The second pig sample of 182 pigs included randomly selected Duroc ($n = 40$) and Yorkshire ($n = 40$) pigs and all available Landrace pigs ($n = 102$). Production traits measured on these animals for the growing period (30–105 kg) included average daily weight gain (kg/day), feed conversion ratio (kg of feed/kg of weight gain), total feed intake (kg), daily feed intake (kg), backfat-EBV (mm, adjusted to

100 kg), age-EBV (days, adjusted to 100 kg), backfat adjusted to 100 kg (mm) and age adjusted to 100 kg (days). This second pig sample was analysed with SAS (SAS Institute Inc. 1989) using weighted ANOVA with the general linear models (GLM) procedure and for each trait, the model included sex and genotype as fixed effects using a stringent threshold for significance ($P < 0.01$).

In the first sample of pigs, there were significant differences in allelic frequencies between positive and negative subgroups of Landrace pigs for the A2845T, G2728A and T3469C polymorphisms (Table 1). These allelic frequencies were higher in the Landrace positive EBV subgroup compared with the Landrace negative EBV group, suggesting a negative effect of these alleles on backfat- and age-EBV. In the Yorkshire breed, the T, C and A alleles at position 2845, 3996 and 2728, respectively, were fixed, whilst the frequency of the C allele at position 3469 did not differ between negative and positive EBV subgroups. Analysis of the second sample indicates that frequencies of the T, C, A and C alleles at positions 2845, 3996, 2728 and 3469, respectively, were low in the Duroc and Landrace breeds whilst the T (2845), C (3996) and A (2728) alleles were absent in Yorkshire pigs, as was found in the first sample (Table 2).

In the second sample, weighted ANOVA revealed an association between the A2845T polymorphism and total feed intake ($P = 0.0061$) in the Landrace breed, with TT homozygotes having an average total feed intake of 218.72 ± 3.87 kg compared with 204.92 ± 1.69 kg for the AA homozygotes. Also in the Landrace breed, an association was observed between the A2845T polymorphism and age-EBV, with TT homozygotes having an average age-EBV of 4.28 ± 0.68 days compared with 1.37 ± 0.47 days for the AA homozygotes. Analyses of the *Hin*fI polymorphism revealed an association between the T3469C polymorphism and average daily weight gain ($P = 0.0078$) for the Landrace breed. TT homozygote pigs had an average daily weight gain of 0.91 ± 0.011 kg/day compared with 0.85 ± 0.019 kg/day for TC heterozygotes pigs. AT heterozygous and CC homozygous estimates are not given for these polymorphisms (A2845T and T3469C, respectively) because only limited animal numbers were available for the statistical analysis.

Although these mutations are not located in the leptin coding sequence, it is possible that they affect mRNA stability or translation efficiency, resulting in specific biological effects. Polymorphisms reported may also act as molecular markers linked to a specific locus which controls growth rate and feed intake traits. The lack of association between production traits and the DNA polymorphisms in the Duroc and Yorkshire breeds suggests that linkage disequilibrium is unique to the Landrace breed. Although all available Landrace pigs ($n = 102$) of the test programme were included in the second sample, a limited number of these pigs carried the deleterious alleles. Moreover, as the polymorphisms seem to be associated with negative production traits (decreased growth rate and increased feed intake), it is not surprising to see low frequencies of these mutant alleles. Indeed, selection pressure which has been maintained to increase pig growth

Table 1 Allelic frequencies of porcine *LEP* for negative and positive subgroups of the first sample of pigs.

Polymorphism	Breed	No. of tested pigs (neg/pos)	Allelic frequency[1] Negative[2]	Allelic frequency[1] Positive[3]	P^4
A2845T	Duroc	26/14	13.5	7.1	0.483
	Landrace	43/13	11.6	34.6	**0.014**
	Yorkshire	39/28	0	0	1
T3996C	Duroc	27/17	3.7	5.9	0.638
	Landrace	44/14	10.2	25	0.062
	Yorkshire	51/27	0	0	1
G2728A	Duroc	28/18	3.6	2.8	1
	Landrace	44/14	9.1	25	**0.048**
	Yorkshire	42/28	0	0	1
T3469C	Duroc	27/17	1.9	2.9	1
	Landrace	44/13	3.4	15.4	**0.046**
	Yorkshire	50/26	8	11.5	0.386

[1] Frequency of T allele for A2845T, C allele for T3996C, A allele for G2728A and C allele for T3469C.
[2] Age EBV ≤ −1.6; backfat EBV ≤ −0.3.
[3] Age EBV ≥ 0.9; backfat EBV ≥ 0.2.
[4] Fisher exact test (two-tailed) probability.
$P < 0.05$ are given in bold.

Table 2 Allelic frequencies of porcine *LEP* polymorphisms and associations with production traits assessed on the second sample of pigs.

Polymorphism	Breed	No. of tested pigs	Allelic frequency		Association between genotype and traits[1]							
			ATGT	tcac	ADWG	FC	TFI	DFI	Backfat EBV	Age EBV	Backfat	Age
A2845T	Duroc	39	0.68	0.32	0.29	0.51	0.74	0.32	0.65	0.08	0.77	0.16
	Landrace	102	0.93	0.07	0.35	0.83	**0.006**	0.61	0.69	**0.003**	0.83	0.52
	Yorkshire	40	1	0								
T3996C	Duroc	40	0.89	0.11	0.42	0.90	0.61	0.45	0.09	0.31	0.39	0.65
	Landrace	102	0.85	0.15	0.84	0.89	0.41	0.75	0.03	0.02	0.20	0.59
	Yorkshire	40	1	0								
G2728A	Duroc	40	0.91	0.09	0.48	0.88	0.81	0.50	0.06	0.49	0.66	0.71
	Landrace	102	0.85	0.15	0.84	0.89	0.41	0.75	0.03	0.02	0.20	0.59
	Yorkshire	40	1	0								
T3469C	Duroc	40	0.91	0.09	0.48	0.88	0.81	0.50	0.06	0.49	0.66	0.71
	Landrace	102	0.94	0.06	**0.008**	0.36	0.28	0.14	0.61	0.33	0.61	0.07
	Yorkshire	40	0.85	0.15	0.39	0.26	0.96	0.99	0.75	0.74	0.47	0.59

[1] Weighted ANOVA probabilities for average daily weight gain (ADWG), feed conversion (FC), total feed intake (TFI), daily feed intake (DFI), backfat EBV, age EBV, backfat (100 kg) and age (100 kg). $P < 0.01$ are given in bold.

rate and decrease backfat thickness over the past 20 years may contribute to the elimination or decrease of unfavourable alleles such as these.

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First Named Inventor	:	Michael E. Spurlock	
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EXHIBIT D

of

DECLARATION

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Hybridization with Radioactive Probes: Using DNA Fragments as Probes.
Current Protocols in Molecular Biology. Section II 6.3.5. (2000)

SECTION II

HYBRIDIZATION WITH RADIOACTIVE PROBES

After plaques or colonies have been transferred to nitrocellulose filters, the desired clone can be detected by its ability to hybridize to a DNA probe. This is a rapid, effective screening procedure that allows the identification of a single clone within a population of millions of other clones. The filters are hybridized with a ^{32}P-labeled nucleic acid probe, the excess and incorrectly matched probe is washed off the filter, and the filter is autoradiographed. Two features of the nucleic acid probe used for these experiments are critical to the successful screening of recombinant DNA libraries. First, the probe must hybridize only to the desired clones and not to any other clones. Thus, the nucleic acid sequence used for a probe must not contain any reiterated sequences or sequences that will hybridize to the vector. Second, the specific activity of the probe must be at least 10^7 cpm/μg. Most of the procedures for labeling DNA or copy RNA molecules are described in Chapter 3, and a support protocol is presented here that allows the 5′ end-labeling of a mixture of oligonucleotides.

The two basic protocols presented in this section describe steps required to hybridize labeled probes to recombinant DNA clones on filters. Two protocols are presented because conditions for hybridizing short oligonucleotide probes and longer nucleic acid probes to filters are different.

UNIT 6.3

Using DNA Fragments as Probes

BASIC PROTOCOL

HYBRIDIZATION IN FORMAMIDE

Bacteriophage plaques or bacterial colonies bound to a filter membrane are detected by hybridization with a radioactive probe. Hybridization proceeds on prewet filters placed in a sealable plastic bag. After hybridization the filters are removed from the sealed bag, excess probe is washed off, and the filters are autoradiographed to identify the clones that have hybridized with the probe.

Materials

Nitrocellulose membrane filters bearing plaques, colonies, or DNA (*UNITS 6.1 & 6.2*)
Hybridization solution I
Radiolabeled probe, 1 to 15 ng/ml (*UNIT 3.5*)
2 mg/ml sonicated herring sperm DNA
High-stringency wash buffer I
Low-stringency wash buffer I

Sealable bags
42°C incubator
Water bath adjusted to washing temperature (see commentary)
Glass baking dish

Additional reagents and equipment for autoradiography (*APPENDIX 3*)

Incubate filters with probe

1. Wet filters with hybridization solution I. Lay a filter membrane bearing plaques on top of 5 to 20 ml of hybridization solution I and allow solution to seep through filter. It is important to wet only one surface at a time to prevent trapping air in filter. Wet each filter in turn, producing a stack of wet filters.

   When multiple filters are to be hybridized to the same probe, no more than twenty 8.2-cm discs or ten 20 × 20 cm square filters should be placed in one stack.

Contributed by William M. Strauss
Current Protocols in Molecular Biology (1993) 6.3.1-6.3.6

Estimate the volume of hybridization solution used to wet the filters; this is a significant fraction of the volume of the hybridization reaction.

2. Transfer the stack of wetted filters to an appropriately sized sealable bag. Add enough hybridization solution to generously cover filters and seal.

   Note the volume of hybridization solution used to cover the filters.

3. Prehybridize filters by placing the bag in a 42°C incubator for at least 1 hr.

   Some investigators omit this step.

4. While filters are prehybridizing, pipet the radioactive probe into a screw-cap tube, add 2 mg (1 ml) sonicated herring sperm DNA, and boil 10 min. Place boiled probes directly into ice to cool.

   The amount of probe used is important, and should be in the range of 1 to 15 ng/ml of hybridization reaction. The volume of the hybridization reaction can be assumed to be the amount of hybridization solution added to the filters.

5. Add 2 ml hybridization solution I to the boiled probe.

6. Remove bag containing filters from the 42°C incubator. Open bag, add probe mixture, exclude as many bubbles as possible, and reseal.

   A good way to add the radioactive probe is to take it up in a syringe with an 18-G needle and then inject it into the bag. Reseal the bag after adding probe.

7. Mix probe in the bag so that filter is evenly covered. Replace bag in the 42°C incubator and let hybridize overnight.

Wash filters to remove nonhybridized probe

8. Warm 1 liter high-stringency wash buffer I to the "washing temperature" in a water bath.

   The stability of washing temperature and salt concentrations are critical features of this experiment. See discussion in commentary.

9. Remove bag containing hybridizing filters from the 42°C incubator. Cut bag open and squeeze hybridization solution out of the bag.

   CAUTION: *Handle material carefully as it is extremely radioactive. This should be done on disposable paper bench covers.*

10. Quickly immerse the filters in 500 ml low-stringency wash buffer I at room temperature in a glass baking dish. Separate all the filters, as they may stick together during hybridization.

    The volume of the low-stringency wash buffer is not important as long as the filters are completely covered. The filters must not be allowed to dry as the radioactive probe will irreversibly bind the filters if the filters dry in contact with probe. (The type of container used to hold the filters is not important as long as it transfers heat well. Thus glass, metal, or enamel containers are better than plastic.)

    The low-stringency wash only removes nonhybridized probe formamide and hybridization solution; it does not determine the stringency of the hybridization.

11. Rinse the filters three times with 500 ml low-stringency wash buffer. Let the filters sit 10 to 15 min at room temperature in low-stringency wash buffer with each rinse.

12. Pour off the low-stringency wash buffer and pour in 500 ml high-stringency wash buffer (prewarmed to washing temperature).

13. Replace the high-stringency wash buffer with another 500 ml of high-stringency wash buffer, then place the glass dish containing the filters in incubator at wash temperature. Make sure that the temperature in the glass dish reaches the desired washing temperature by placing a thermometer directly into the bath and measuring the temperature. Usually 15 to 20 min at the desired wash temperature is sufficient to remove most of the background radioactivity.

    Of course, if the glass dish is placed in a water bath, be careful that the water from the water bath does not get into the filters.

Autoradiographing filters

14. Remove filters and mount them either wet or dry on a plastic backing. If the filter(s) is to be exposed wet, then isolate it from the film by covering it with plastic wrap.

    Used X-ray film provides a good form of plastic backing for filters.

15. Mark the filters with radioactive ink to assist in alignment and autoradiograph.

    An easy way to apply radioactive ink is to mark adhesive-backed paper labels with radioactive ink and then attach the stickers to the plastic wrap cover.

    X-ray intensifying screens greatly decrease the amount of exposure time required.

ALTERNATE PROTOCOL

HYBRIDIZATION IN AQUEOUS SOLUTION

This method differs mainly in that formamide is not used in the hybridization solution. Follow the basic protocol except use the reagents and alternate parameters listed below.

Additional Materials

Hybridization solution II
Low-stringency wash buffer II
High-stringency wash buffer II
65°C incubator

1. Prehybridize as in basic protocol except that the filters are prehybridized at 65°C using hybridization solution II.

    Hybridization solution II may have to be prewarmed to solubilize the SDS.

2. Prepare probe as in step 4 of basic protocol and dilute with 2 ml of hybridization solution II.

3. Hybridize overnight as in steps 6 and 7 of basic protocol except use a hybridization temperature of 65°C.

4. Remove bag containing hybridization from the 65°C incubator. Squeeze out the hybridization solution, taking care to avoid contamination with the excess radioactive hybridization solution.

5. Immediately rinse filters twice with low-stringency wash buffer II.

    It is unnecessary to maintain a given temperature for this wash; just let the filters sit in wash buffer at room temperature until ready to proceed.

6. At 65°C, proceed to wash filters with high-stringency wash buffer II. Employ multiple quick washes (5 to 8) and immerse filter in a final wash for ~20 min. Check the radioactivity of the filters with a Geiger counter and be certain that they produce a signal only a fewfold above background levels.

REAGENTS AND SOLUTIONS

High-stringency wash buffer I

0.2× SSC (APPENDIX 2)
0.1% sodium dodecyl sulfate (SDS)

High-stringency wash buffer II

1 mM Na_2EDTA
40 mM $NaHPO_4$, pH 7.2
1% SDS

Hybridization solution I

Mix following ingredients for range of volumes indicated (in milliliters):

Formamide	24	48	72	120	240	480
20× SSC	12	24	36	60	120	240
2 M Tris·Cl, pH 7.6	0.5	1.0	1.5	2.5	5.0	10
100× Denhardts solution	0.5	1.0	1.5	2.5	5.0	10
Deionized H_2O	2.5	5.0	7.5	12.5	25	50
50% dextran sulfate	10	20	30	50	100	200
10% SDS[a]	0.5	1	1.5	2.5	5	10
Total volume	**50**	**100**	**150**	**250**	**500**	**1000**

[a]In place of SDS, *N*-lauroylsarcosine (Sarkosyl) may be used.

Add the SDS last. The solution may be stored for prolonged periods at room temperature.

The dextran sulfate should be of high quality. Pharmacia produces acceptable-grade dextran sulfate. Recipes for SSC and Denhardt's solution are in APPENDIX 2.

Hybridization solution II

1% crystalline BSA (fraction V)
1 mM EDTA
0.5 M $NaHPO_4$, pH 7.2 (134 g $Na_2HPO_4 \cdot 7H_2O$ plus 4 ml 85% H_3PO_4/liter = 1 M $NaHPO_4$)
7% SDS

Low-stringency wash buffer I

2× SSC (APPENDIX 2)
0.1% SDS

Low-stringency wash buffer II

0.5% BSA (fraction V)
1 mM Na_2EDTA
40 mM $NaHPO_4$, pH 7.2
5% SDS

Sonicated herring sperm DNA, 2 mg/ml

Resuspend 1 g herring sperm DNA (Boehringer Mannheim #223636) in a convenient volume (about 50 ml of water) by sonicating briefly. The DNA is now ready to be sheared into short molecules by sonication. Place the tube containing the herring sperm DNA solution in an ice bath (the tube must be stable even if the ice begins to melt). The sonicator probe is placed in the DNA solution (without touching the bottom of the vessel). The sonicator is turned on to 50% power 20 min, or until there is a uniform and obvious decrease in viscosity. At no time should the tube containing the DNA become hot to the touch. After sonication, the DNA is diluted to a final concentration of 2 mg/ml, frozen in 50-ml aliquots, and thawed as needed.

COMMENTARY

Background Information

All hybridization methods depend upon the ability of denatured DNA to reanneal when complementary strands are present in an environment near but below their T_m (melting temperature). In a hybridization reaction involving double-stranded DNA on a filter and a single-stranded DNA probe there are three different annealing reactions occurring. First, there are the desired probe-DNA interactions which result in signal. Second, there are mismatch interactions that occur between related but nonhomologous sequences; these mismatch hybrids are the ones that must be eliminated during the washing of the filters. Non-sequence-specific interactions also occur and these result in noise. The ability to extract information from a particular hybridization experiment is a function of the signal-to-noise ratio. High background or poor specific signal can both result in uninterpretable results.

Washing nitrocellulose filters is required to remove excess radioactive probe, as well as radioactive probe that has bound to the DNA on the filter as mismatch hybrids. Temperature and salt concentration dramatically affect the maintenance of specific hybrids. Detergents and other charged species can have a profound effect upon the nonspecific binding of species that contribute to background. In this protocol, hybridization is achieved in a solution containing 50% formamide. Excess probe is rinsed away under low-stringency conditions so that further hybridization will not occur. Once the hybridization solution is rinsed away, it is possible to proceed to a high-stringency wash without fear of further hybridization. When washing is complete, the filters should produce very little "noise" when monitored with a Geiger counter. Although single-copy sequence probe normally does not produce a signal that is detectable with a Geiger counter, a probe corresponding to more abundant sequences will produce a signal that can be "heard" with a Geiger counter.

Literature Review

Hybridization to filter membranes forms a basis of recombinant DNA technology and is described in detail earlier in the manual (UNIT 2.9). The protocols described here vary from those used for Southern blot filter hybridization in that the volume of the hybridization is usually larger and the washing conditions are different. Dextran sulfate is an important component of the hybridization solution as it increases the rate of reassociation of the nucleic acids.

The protocols in this unit describe methods for hybridizing radioactive probes to membrane-bound plaques or colonies. These procedures for screening recombinant clones were first suggested by Grunstein and Hognes (1975) and by Benton and Davis (1977). The conditions of hybridization proposed in the basic protocol involving hybridization in formamide was originally described by Denhardt (1966) and Gillespie and Spiegelman (1965) while the alternate protocol using aqueous hybridization solution was introduced by Church and Gilbert (1984).

The method of washing filters under stringent conditions to remove background was first proposed by Southern (1975). Botchan et al. (1976) described the benefit of adding SDS to the wash solution. Jeffreys and Flavell (1977) first employed the wash conditions described in the protocols presented here.

Critical Parameters

Hybridization. Kinetically, the hybridization of DNA (or RNA) probes to filter-bound DNA is not significantly different from hybridization in solution. For single-stranded probes, the rate of hybridization follows first-order kinetics, since probe is available in excess. Under conditions of excess probe, the time for hybridization is inversely proportional to the probe concentration. For double-stranded probes the rate of hybridization displays a more complex relationship to the initial probe concentration. However, to a first approximation the initial probe concentration is inversely proportional to the rate of hybridization. To determine the actual time required for the successful hybridization of a given probe, either empirical data must be available or the following formula can be used to determine the length of time (in hours) required to achieve 50% hybridization (T_{50}):

$$\frac{1}{x} \times \frac{y}{5} \times \frac{z}{10} \times 2 = T_{50}$$

where x is the weight of probe in micrograms; y is the complexity of probe in kilobases; and z is the volume of hybridization solution in milliliters. The length of time T is given in hours. Maximum hybridization signal will be obtained if the reaction is allowed to proceed to $5 \times T_{50}$, although 1 to $2 \times T_{50}$ is often used.

It is also clear that nonspecific interactions

occur and that in any hybridization, sources of noise will be present. Therefore, from a practical standpoint one conventionally utilizes concentrations of nick-translated probe on the order of 1 to 15 ng/ml of hybridization, where the specific activity of the probe is from 5×10^7 cpm/µg to $>10^8$ cpm/µg. Too much probe in a hybridization is as bad as too little.

One important source of background hybridization to filters is due to the hybridization of the probe to vector sequences or to *E. coli* DNA. Be certain that there is no vector or *E.coli* DNA sequences in the probe. This can best be ensured by isolating the probe from one type of vector (e.g., plasmid) and screening a library made with a different type of vector (e.g., bacteriophage).

Washing temperature. Washing at low stringency is a straightforward proposition. Buffer is added at room temperature and washing proceeds at room temperature.

High-stringency wash is determined empirically. The relative homology between the probe and target sequence is a determining parameter. If the homology is 100%, a high temperature (65° to 75°C) can be used. As the homology drops, lower washing temperatures must be used. In general one starts at 37° to 40°C, raising the temperature by 3° to 5°C intervals until background is low enough not to be a major factor in the autoradiography.

The length of the probe is also important. Very short probes (<100 bp) must be washed at lower temperatures, even if the homology is 100%. Washing strategy is the same as for probes of differing homology.

Salt concentration. The lower the salt concentration, the higher the stringency. With this said, the protocols as outlined do not require adjustment of salt concentration for adjustment of stringency. Only the washing temperature is varied.

Probe. The nucleic acid probe must be of high specific activity and greater than 50 bp in length so that it can form stable hybrids.

Anticipated Results

After washing the filters the background should be barely detectable with a Geiger counter.

With a high-specific-activity probe $>5 \times 10^7$ cpm/µg and an overnight hybridization reaction with a 1-kb unique sequence probe, hybridizing bacterial colonies or bacteriophage plaques can be visualized after a 1 to 18 hr exposure.

Time Considerations

Generally hybridizations are carried on overnight for 12 to 16 hr. This is sufficient for most probes and blots. However, with probes of increasing complexity longer hybridization times are required. This is preferable to increasing the probe concentration from experiment to experiment.

Autoradiography requires 1 to 18 hr.

Literature Cited

Benton, W.D. and Davis, R.W. 1977. Screening λgt recombinant clones by hybridization to single plaques in situ. *Science* 196:180.

Botchan, M., Topp, W., and Sambrook, J. 1976. The arrangement of simian virus 40 sequences in the DNA of transformed cells. *Cell* 9:269-287.

Church, G. and Gilbert, W. 1984. Genomic sequencing. *Proc. Natl. Acad. Sci. U.S.A.* 81:1991-1995.

Denhardt, D. 1966. A membrane filter technique for the detection of complementary DNA. *Biochem. Biophys. Res. Commun.* 23:641-646.

Gillespie, D. and Spiegelman, S. 1965. A quantitative assay for DNA–RNA hybrids with DNA immobilized on a membrane. *J. Mol. Biol.* 12:829-842.

Grunstein, M. and Hogness, D. 1975. Colony Hybridization: A method for the isolating of cloned DNA's that contain a specific gene. *Proc. Natl. Acad. Sci. U.S.A.* 72:3961.

Jeffreys, A.J. and Flavell, R.J. 1977. A physical map of the DNA region flanking the rabbit β globin gene. *Cell* 12:429-439.

Southern, E.M. 1975. Detection of specific sequence among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503-517.

Contributed by William M. Strauss
Harvard Medical School
Boston, Massachusetts

First Named Inventor	:	Michael E. Spurlock	
Appln. No.	:	09/928,522	
Filed	:	August 13, 2001	Group Art Unit: 1647
Title	:	Bovine Leptin Protein, Antisense and Antibody	Examiner: C. J. Saoud
Docket No.	:	LL31.12-0015	

EXHIBIT E

of

DECLARATION

submitted under 37 C.F.R. 1.132

Course Instructions posted at http://catlserver.tamu.edu/ning/ing626.htm for Lab Session No. 6, Fall 2003: Course Entitled Gene Expression (ANSC/GENE 626 (edited by. N. Ing 9/2/03)); Nancy H. Ing, Instructor; Texas A & M University; College Station, Texas

Fall 2003: GENE EXPRESSION
ANSC/GENE 626 (edited by. N. Ing 9/2/03)
Nancy H. Ing, Instructor
Kleberg 410D, 862-2790
ning@cvm.tamu.edu

The purpose of this course is to provide graduate students with experience in working with RNA and DNA and with the theories behind the use of molecular biology in research.

Class will be held in BICH 243 in Fall semester on Thursdays (lecture from 12;40 to 1;35 p.m.) and Fridays (lab from 12:45 to 3:40 p.m.)

9/04	Lecture 1:	Introduction to the course, Safety rules What is gene expression? What is a gene? What does it do?
9/05	Lab 1	Introduction: Pipetting, Restriction digestion of plasmid DNA.
9/11	Lecture 2	What are plasmids and how are they used? Electrophoretic analysis of macromolecules.
9/12	Lab 2.	Plasmid DNA analysis by agarose gel electrophoresis
9/18	Lecture 3	Engineering and amplifying DNA in basic and specialized vectors
9/19	Lab 3.	Ligation of gel-purified DNA fragments
9/25	Lecture 4:	Growing and preparing plasmid DNA. Other DNA preps.
9/26	Lab 4	Transformation of E. coli
10/2	Lecture 5:	Restriction enzymes and analysis of plasmid DNA
10/3	Lab 5.	Plasmid minipreps
10/9	Lecture 6:	How can you identify a DNA specifically? (two ways)
10/10	Lab 6:	Restriction analysis of plasmid DNA minipreps
10/16	Lecture 7:	What does DNA sequence tell you? Functions of gene sequences
10/17	Lab 7	DNA sequencing with PCR
10/23	Lecture 8	Transcription in cells and out
10/24	Lab 8:	Analyzing DNA sequences
10/30	Lecture 9	What natural types of RNA are in cells? How do you analyze RNA? What are their functions?
10/31	Lab 9:	Making a DNA template for in vitro transcription of an sense RNA
11/6	Lecture 10:	Acrylamide gel electrophoresis for small RNA and protein analyses
11/7	Lab 10	In vitro transcription and PAGE analyses
11/13	Lecture 11	Translation in cells and in vitro

11/14	Lab 11:	In vitro translation and making SDS-PAGE gels
11/20	Lecture 12	Analyses of proteins by SDS-PAGE
11/21	Lab 12	Running protein samples on SDS-PAGE and staining
11/27 and 28		HAPPY THANKSGIVING!!!
12/ 4	Lecture 13	Other Analysis of proteins
12/5	Lab 13:	Destaining and Analyzing the SDS-PAGE results.
11/21		11A. Analysis of Extracted RNA by A260; In vitro transcription of cRNA probes Overnight cultures

13. Plasmid DNA restriction and gel analysis

Protocols will be provided. Required viewing of videos. Required reading from Gerstein "Molecular Biology Problem Solver" as well as other materials relating to kits, etc used. Students will be evaluated on preparation and participation (33%), lab notebook (33%) and a written laboratory report (33%) due the Friday after the last class.

ANSC/GENE 626 REQUIRED READING & VIDEOS

- Required Text: Gerstein "Molecular Biology Problem Solver" 2001
- Lab 1 Pipettors pp 67-77.
- Lab 2 RNA purification pp 197-224
- TriPure handout - see Roche website at http://biochem.boehringer-mannheim.com
- Isolation of mRNA Video*
- Lab 3 SPectrophotometers pp 94-111
- Ambion Maxiscript and Northernmax (formaldehyde gel) Protocols - see ambion website at www.ambion.com
- Lab 4 Gel Electrophoresis pp331-371
- Polyacrylamide Gel Electrophoresis Video
- Lab 5 Nucleic acid hybridization pp 399-460
- Lab 6 DIG RNA labeling kit instructions from Roche (see website biochem.roche.com)
- Southern Blot Hybridization Video
- Lab 7 DIG luminescent detection kit from Roche
- Lab 8 Clontech MIMIC kit information
- Polymerase Chain Reaction pp291-330
- PCR and Detection Video
- Lab 9 Agarose Gel Electrophoresis Video
- Lab 10 DNA Ligation Video
- Lab 11 Transformation Video
- Lab 12 DNA Purification pp167-195
- Rapid Isolation of Plasmid DNA (minipreps) Video
- Lab 13 Restriction enyzymes pp225-266

*Videos may be checked out from Kleberg 410A for 1 h during the day or overnight after 4PM (returned by 9AM). Single and group viewing is available in the Multi Media Center (Kleberg 023).

LAB RULES

A. GENERAL

1. Everyone is individually responsible for the experiments. Come prepared by reading protocols and required reading in advance!! Activities will be started immediately, while explanations and discussion sessions will occur as time permits. COME PREPARED to ASK QUESTIONS, especially during discussions.

2. Equipment in this and neighboring labs is shared. Know or ask how to use it. Obey user rules, such as signing logs. Leave all equipment in good working order. If there are problems, tell someone so we can fix them!

3. Leave the lab better than you found it. Wash your own glassware, clean up your work area, write the names of reagents that are running out on the "to be ordered" list, etc.

4. Lab notebooks are bound volumes, are kept in pen with numbered, dated pages. They are designated only to that purpose, are labelled on the cover with Name, dates and lab, and are only removed from the lab with instructor permission. In them, each person describes their activities and observations each day. Each page should be dated. Each photo or X-ray film should be labeled with initials, date, and identification of gel type, lane contents and dye migration. Record things chronologically along with dates. Start a new project on a new page with a description of its purpose. A stranger should be able to pick up your notebook and understand how and why you did an experiment. Protocols don't have to be written each time, but may be referred to, instead. For this, you may consider the handouts you receive in lab as references. Use them as such and refer to specific pages (dates) within them. Record your activities and observations clearly, using complete and understandable sentences. Write down your reagents, including buffers and buffer recipes. KEEP UP WITH NOTEBOOK ENTRIES EVERY DAY...otherwise, data will be lost!

5. All reagents and samples saved must be labeled with the date; your initials, and WHAT IT IS. In my lab, we use simple sample numbers, such as 1 - 10. To key it into our notebook, we write: lab notebook #, page #; (NI2P30 relates to Nancy Ing Book 2, page 30), so the notebook, not the tube, is where the full description of the sample exists. Items not labeled sufficiently may be discarded.

6. Store things in appropriate places! For plasmids and reactions and buffers, store at –20C in storage box provided to your group unless otherwise noted. Note storage places in your notebook.

B. SAFETY IS THE #1 PRIORITY

1. The only safety activity not strictly enforced is wearing safety glasses: this is a good idea but is not mandatory. Wearing a lab coat is mandatory and wearing gloves will become a habit (see below).

2. Working with open flames and hazardous chemicals have strict safety protocols - ask for them and follow them.

3. Working with radioactivity is a privilege, not a right. Workers must monitor for contamination, before, during, and after the procedure. Radiation safety training is required. WE RUN A CLEAN LAB.

4. Because we work with HAZARDOUS SUBSTANCES, there is NO EATING, DRINKING, SMOKING, or APPLYING MAKE-UP in the lab.

5. Garbage must be disposed of properly. Glass and sharps, biohazard, chemical, and radioactive waste must be separated from the rest.

C. GOOD LAB TECHNIQUES

1. ICE IS NICE! Work on it unless otherwise directed. It slows degradation of macromolecules.

2. Many reagents settle on storage, so mix them! All frozen solutions need to be thawed and mixed before using.

3. ENZYMES DO OUR WORK. They are stable as glycerol solutions at -20?C. Keep them in the freezer as much as possible. Only remove them in -20oC blocks. DO NOT WARM ENZYME STOCKS! When pipetting small amounts of viscous solutions like enzymes, check loaded pipet tip and evacuated one to assure that enzyme got into the reaction. After addition, mix reaction solution gently but thoroughly: can pipet total volume up and down OR vortex gently and flash spin in microfuge to return reaction to the tube bottom.

ADA Statement, Copyrights, and Plagiarism

The Americans with Disabilities Act (ADA) is a federal antidiscrimination statute that provides comprehensive civil rights protection for persons with disabilities. Among other things this legislation requires that all students with disabilities be guaranteed a learning environment that provides for reasonable accomodation of their disabilities. If you believe you have a disability requiring an accomodation, please contact the Dept. of Student Life, Services for Students with Disabilities in Room 126 of the Koldus Bldg. or call 845-1637.

Copyrights

The handouts used in this course are copyrighted. By handouts", I mean all materials generated for this class, which include but are not limited to syllabi, quizzes, exams, lab problems, in-class materials, review sheets, and additional problem sets. Because these materials are copyrighted, you do not have the right to copy the handouts unless I expressly grant permission.

Plagiarism

As commonly defined, plagiarism consists of passing off as one's own ideas, words, writings, etc., which belong to another. In accordance with this definition, you are committing plagiarism if you copy the work of another person and turn it in as you own, even if you should have the permission of that person. Plagiarism is one of the worst academic sins, for the plagiarist destroys the trust among colleagues, without which research cannot safely be communicated.

If you have any questions regarding plagiarism, please consult the latest issue of the Texas A&M University Student Rules, under the section "Scholastic Dishonesty."

BICH/GENE 432

Safety Precautions

1. ABSOLUTELY no food or drink in the lab. This includes gum.

2. Always wear gloves when working with Ethidium bromide, radioactive compounds, acrylamide and organic compounds.

3. Always wear UV safety glasses when using UV illumination.

4. Use special care when working with open flames. Don't forget to turn off gas after use.

5. Clean up spills immediately. Notify instructor if hazardous compounds are spilled.

6. Discard organic solutions in appropriate bottles.

7. Discard Ethidium bromide waste in correct container.

8. All culture medium and labware used for bacteria needs to be autoclaved or put in Chlorox before disposal or washing.

9. After using radioactive materials, wash the work area and clean spills immediately. Dispose of gloves in radioactive waste as soon as you are finished.

10. All sharps (including broken glass, needles and razor blades) should be disposed in clearly marked containers, not in the general trash.

11. If you have questions about anything, ASK!

12. Lab coats are required!

LAB REPORTS - BICH/GENE 432
by Linda Guarino

TITLE - This is the most important part of a manuscript. A reader begins here, and will also finish here if the title does not promise a subject of interest to him/her. A good overall rule is to use the fewest possible words that adequately describe the contents of the paper. But, do not sacrifice words for specific information. For example 'DNA cloning' is a short title, but it is too general. A popular trend in recent years is to publish papers where the title is a complete sentence that summarizes the major conclusion of the manuscript. Personally, I prefer titles that describe the work, not the results.

ABSTRACT - An abstract is a mini version of the paper. It should provide a brief (less than 250 words) summary of the major points of the manuscript. The abstract should state the objectives, describe the methodology used, summarize the results, and state the principle conclusions. The abstract should be written in the past tense, because it refers to work done.

INTRODUCTION - First of all, state the nature and scope of the problem investigated. Review the pertinent literature(NOT NECESSARY FOR THIS CLASS). Describe the method of the investigation. State the principle results. State the principle conclusions suggested by the results. The first two parts should be in present tense, while comments relating to the present study should be in past tense.

METHODS - The methods section should expand upon the description of the methodology that was presented in the abstract. The order of presentation is usually chronological (methods used in initial stages of the study are presented first). However, sometimes it makes more sense to group similar methods into sections, even though they were not used at the same time. Due to space limitations in journals, methods are not usually described in detail if they have previously been published. If a scientist uses a protocol that is identical to one previously described, he/she would state 'The DNA was prepared according the procedure previously described (reference). If there were minor differences, he/she would state 'according to the procedure of (ref.) with minor modifications' and then describe the modifications. In this class, you may assume that the class protocol has been published. Therefore you don't need to give the details, but you need to describe the general strategy. For example, you should say 'the DNA was purified by the alkaline lysis procedure as previously described' not 'the DNA was purified as previously described'. In addition to the class protocol, you could also reference the Cloning manual or the Promega manual. The methods section should be written in past tense.

RESULTS - The results section is a presentation of the data. It should not repeat the methods given in the previous section. Each figure should be referred to here. The results section should be written in past tense.

DISCUSSION - The discussion should put the results into perspective. Discuss the results without recapitulating the results section. Show how your results and interpretations agree. State your conclusions clearly, and summarize the evidence for each conclusion. Selection of correct tense is more difficult in the discussion than in the other sections. Your own work should be described in past tense. If reference is made to published work, it should be in present tense.

REFERENCES - Only need to cite the class protocols and any other sources of material...no need for literature review so these are very few.

FIGURES and FIGURE LEGENDS - Present the important data in figure form, raw data if possible for this class. Figure legends begin with a title for the figure. Figures should have complete legends - so that they can be understood without reading the rest of the paper. These may be nested in the paper or placed at the back.

Many students have asked about length. The best rule that I can give you is that it should be long enough to convince me that you have learned something. However, I have a short attention span, and if your paper is very long and verbose, I may lose interest before I decide whether or not you have learned anything.

Suggested reading:

Day, R. A. 1988 How to write and publish a scientific paper, 3rd ed. Oryx Press, Phoenix.

9/6/02

Introduction to Gene Expression

General

The purpose of this class is to provide motivated students with the beginning skills required to apply molecular biology techniques. Protocols and hands-on exercises will teach several techniques, but more importantly, will allow new procedures to be mastered during subsequent research projects. Students will learn how to handle nucleic acids through basic purification and measuring and manipulative procedures. However, comprehensive coverage of the underlying biochemistry of DNA modulatory enzymes, for example, is not possible in the time allotted. Excellent courses exist for such background studies (GENE 431 and 450), and required reading and suggested references will fill those voids. Brief discussions of how applications of molecular biology are used in physiological studies will be held as time permits. Questions are encouraged, also. We will have free time during incubation periods - use these wisely with questions, reading or writing for your notebook or lab report.

The major criticism of this course is that the flow of experiments overlaps. This is life. Experiments proceed too slowly with long incubation times to just sit and wait. It's up to us to keep the purpose of the experiments in our minds (and notebooks!).

This course is designed to help new students study expression of their favorite gene in their favorite tissue. For this class, your favorite genes are actin and glyceraldehyde phosphate dehydrogenase (GAPDH), because they are highly expressed in your favorite tissue (endometrium.) Genes are transcribed into messenger RNAs, so first we'll:

1) Extract RNA from tissue and use it to make a Northern blot. The amount of RNA obtained is measured by sample absorbance at 260mm. To assess RNA quality, the RNA preparation is analyzed on a denaturing gel. This is transferred to a membrane ("Northern blotting") for hybridization with probes for specific mRNAs (actin or GAPDH)..

2) Make a cRNA probe for actin or GAPDH mRNA and hybridize it to the tissue RNA on the Northern blot. The lab boss gives out plasmid clones containing complementary DNAs (cDNA's, synthetic copies of fragments of mRNAs). The best probes are cRNAs. To make them, the circular plasmid DNA is restricted or cut at a specific site with a restriction enzyme. For in vitro transcription, the plasmid and ribonucleotides are combined with a bacteriophage RNA polymerase (SP6 or T7). The polymerase enzyme binds a specific site on the plasmid and transcribes (makes RNA) using the DNA as a template. Either of the two strands of the DNA can be reproduced as cRNA: the top cDNA strand is like mRNA and, if transcribed, is called "sense" cRNA. The sense cRNA is useful as a template for translation. The bottom cDNA strand is complementary to the sense strand and hybridizes to mRNA, as does its transcription product called "antisense" cRNA. We'll only synthesize the antisense cRNA and it will be labeled with Digoxigenin so it will be a probe (detectable reagent) for identifying its

homologous RNA in the tissue RNA samples. After hybridizing the probe to the RNA on the Northern blot and washing the blot, specifically bound probe will be detected with DIG-antibody and chemiluminescent detection reagents.

3) Quantitate estrogen receptor mRNA with RT-PCR. But what if you only have a very small amount of tissue and/or the mRNA you want to study is rare (such as estrogen receptor (ER))? The most sensitive mRNA quantitative technique is quantitative Reverse Transcriptase-polymerase chain reaction (PCR). Reverse transcriptase copies RNA into cDNA. The reverse transcription of tissue RNA will provide cDNA that will be used to amplify a specific target cDNA between two previously designed primers Since PCR is fickle, the best way to make it quantitative is to use an internal control DNA that competes for the same primers and reagents but can be distinguished (by slightly different size on agarose gels). Therefore, one runs the PCR reactions on an agarose gel and simply looks for the lane with equivalent bands (PCR products) from the internal control and target cDNAs in a titration set of PCR reactions. Since product amounts are the same, starting material must be the same; thus mRNA concentration = internal control concentration, the latter of which is known for that reaction.

4) Subclone beta-globin cDNA to a more useful plasmid vector. But what if the cDNA clone you have is in a plasmid vector that doesn't have SP6 or T7 RNA polymerase sites (many old plasmids don't). You may have to sub-clone the cDNA fragment into your desired vector. The easiest way utilizes PCR to amplify the cDNA. Restriction enzyme sites can be created by synthesizing them on the ends of the primer. This allows easy insertion and ligation of the cDNA and vector. The new plasmid is forced into E. coli cells during transformation, where presence of the plasmid confers a new phenotype to the bacteria: resistance to the antibiotic ampicillin. Clonal colonies are grown and their plasmid DNA prepared (mini-preps) individually to identify the desired clone (by restriction enzyme analysis).

Thus, our different lab exercises all fit together into common techniques utilized in studies of gene expression. KEEP THE FLOW OF LOGIC IN MIND! Don't just come in and mix reagents and shuffle tubes. Think of the molecules and what you want to learn from them. Predict (visualize) the results of your experiments before you perform them.

YOUR SUCCESS DEPENDS ON PREPARATION:

You must read and think through the experiments BEFORE THE LAB to be able to perform and interpret them well. Note that grades are 33% preparation!

Lab 1 9/6/02

Preparing Materials for RNA Work

Beating RNase

You need to read about RNase, a ubiquitous enzyme that efficiently destroys RNA. Primarily, this will serve to make you paranoid and do neurotic things, like wear gloves all the time. Although working with RNA is similar to working with DNA, many RNA experiments fail miserably because of RNase, so know this enemy!

In biochemistry, RNase is the model of an enzyme that will not die: not in an autoclave or even after dehydration (by alcohol, etc.). As soon as it returns to a water environment between room temperature (R.T.) and 37oC, it chews again. It is an enzyme of all living things and is important in keeping RNA turnover high so cells don't choke on RNA and so new expression of genes tightly regulates cell function.

The best way to beat RNase is to avoid it. Work with the cleanest reagents and lab-ware. Things that aren't handled by living thing are generally RNase-free; e.g. paper towels. Test tubes and pipettes don't have to be sterile but should be used from freshly opened packages. Then protect packages from dust and fingers by resealing packages and storing in cabinets. Glassware is reserved similarly: wrapped and stored away from general use. Equipment like Pipettemen and Gel apparati for RNA are reserved for this use and are NOT USED WITH RNase!

Solutions are made with water of the highest purity. Dry chemicals are shaken out of containers: residual amounts are discarded. Nothing dirty is introduced into chemical stocks: solutions or powders.

All solutions are treated with 0.1% diethyl pyrocarbonate (DEPC). This oily liquid is added. The solution is shaken vigorously until foamy (aerobic workout). The solution is incubated 37oC overnight to allow the DEPC to covalently attack RNase. The solution is then autoclaved to destroy DEPC (which also attacks RNA) and to sterilize to prevent growth of undesirables. Exceptions to this solution preparation protocol are 20% SDS (nothing grows in this) and Tris solutions (which DEPC attacks, too). NOTE: DEPC treatment can only correct a low level of RNase contamination! You must start clean!!!

Today's Exercise

Apply benchcote and work with gloves on and tubes/racks on top of diapers.

A. Pack tips: 1 blue box and 2 yellow boxes per group

1. Pour clean blue tips onto a clean surface (paper towel or diaper).

2. With new gloves, pack tips.

3. Write name on box & protect it!

4. Repeat with yellow tips: fill 2 boxes.

B. Practice pipetting

1. Read instructions for pipetting in Appendix.

2. Tare a 1.5 ml test tube on the balance.

4. Weigh 1000 ul of water two times.

5. Repeat with Isopropanol.

6. Determine the densities of these liquids.

7. Measure the volumes of the unknown samples provided in the 1.5ml test tubes.

8. Check your results with an instructor.

C. Each person should test tap water, distilled and DEPC-treated water for Rnase:

1. Label 4 Rnase-free 1.5 ml tubes. Pipet 4 ul of either tap, distilled or DEPC H2O into each (two tubes get DEPC-H2O).

2. Pipet 1ul test RNA into each tube.

3. Put all but one DEPC-H2O tube in 37oC block for 1 h.(The lone DEPC-H2O tube should be kept on ice during the 1 h incubation.)

4. Store all of the tubes at -80oC until Lab 4.

D. Make 1 li DEPC-H2O and 1 li of 20XSSC per person

1. In 1 liter bottle add nanopure H2O to 1 liter level for DEPC-H2O. For 20X SSC, add 175 g NaCl and 88 g Na citrate to a 1 li bottle and dissolve in nanopure H2O.

2. Add 1ml DEPC per liter.

3. Shake till foamy for 10 sec.

4. Put in 37oC incubator O/N to allow DEPC to work optimally.

Lab 2 9/13/02

RNA Extraction

[Autoclave the DEPC-H2O to destroy DEPC and prevent any growth in solutions that might introduce RNase.NOTE on Autoclaving: Need 35 to 40 min. sterilization time for 1 liter. 20 min. for 500ml. Use "liquid" cycle and keep caps loose]

You know how to fight RNase to keep materials clean. GUESS WHAT! RNase is in all living systems including the one from which you'll purify RNA. So all RNA preparers begin with the realization that their worst enzyme enemy is present in the sample. In the cell, RNA is compartmentalized away from RNase so many tissues are OK for harvesting for RNA if kept cool 2-6 h after collection (of course, faster may be better). But freezing breaks intra-cellular membranes, mixing RNA with RNase. Therefore, fresh tissues are kept cool while mincing and

weighing, then are put in a 1.5 ml polypropylene tube snap frozen in liquid N2. They are stored at -80?C. They may store well for 6 months but usually not for 1-2 years. This is dependent on them never thawing, too. So the TWO MAIN POINTS about tissue collection are to SNAP FREEZE and KEEP at -80oC until use within 1 year. NOTE: You can't snap freeze things much bigger than 0.5 cm3. I mince to about 5 mm or less. Tissues vary with RNase content and amount of connective tissue present, so RNA yields vary in quality and amount. RNA extraction from cultured cells results in very high quality RNA, usually.

RNA Extraction from tissue with Boehringer Mannheim TriPure reagent (contains phenol! see NOTE 1 below)

Each student will do 2 RNA preps (one from endometrium, one from spleen). Label all tubes needed NOW!

1. Homogenize 0.5 mg tissue (frozen or fresh) in 5 ml room temperature ("RT") Tripure solution in a 50 ml polypropylene tube. Use three 15 sec bursts at 70% power. Rinse probe in tripure (do a mock homogenization with Tripure and no tissue) between dissimilar samples.

2. Incubate RT 5min. During this time, transfer the contents equally into 4 - 1.5 ml tubes.

3. Add 250 ul chloroform using a P-1000. Mix by vortexing or shaking vigorously 15 sec.

4. Incubate RT 5 min.

5. Centrifuge 15 min at 10,000 rpm at RT or 4oC in a microfuge.

6. Transfer upper phase to four clean 1.5 ml tubes with transfer pipet. AVOID THE INTERFACE!!!! Discard lower phase and interface in phenol waste container.

7. Precipitate RNA by adding an equal volume of isopropanol. Mix by inverting tube. Incubate RT 5 min

8. Centrifuge at 10,000 rpm for 10 min at RT.

9. Wash pellet in 75% EtOH (make 10 ml with 100% EtOH and DEPC H20). This means to discard the supernatant, add the supernatant volume of wash (75% EtOH), vortex, microfuge 5 min, and discard supernatant. The purpose is to wash salts out of the RNA pellet, which should not dissolve during the procedure.

10. Air dry pellet briefly after spin. You can wipe the sides of the tubes with Kimwipes, but stay away from the pellets! Do not dry totally or you will not be able to solubilize RNA easily!!!

11. Store pellet at -80oC.

NOTE 1: Tripure has phenol and guanidine salts in it...both are caustic and burn skin!!! Be careful! Wear safety glasses!!!

NOTE 2: CHCl3 (Chloroform) dissolves things like styrofoam and polystyrene - use glass graduated pipets and polypropylene 15 & 50 ml tubes.

BEFORE YOU LEAVE, CLEAN UP AND RECORD ACTIVITIES IN NOTEBOOK!!!

NOTE 3: Record observations in notebook!

Examples:1) Lysate in step 2 was viscous!

2) Tube #2 fell and was lost.

3) RNA pellet #5 took 20 min. to dissolve, while #1 took only 1 min.

Lab 3 9/20

Analyzing Extracted RNA by Absorbances; In Vitro Transcription of cRNA Probes

A. Solubilize RNA samples from tissue and measure Absorbance at 260 nm and 280 nm

Absorbance measures of DNA & RNA at 260 nm are used to estimate concentrations of nucleic acids. An Absorbance of 1.0 for solution of double-stranded (ds) DNA has =50 ug/ml while RNA has A260 =40 ug/ml and single-stranded (ss) DNA A260 =37 ug/ml. An unknown sample of RNA can be measured for A260 and [RNA] = A260 X dilution factor X 40

The ratio of A260/A280 is an indication of the purity of the nucleic acid. The ratio for pure aqueous DNA is 1.8 while for RNA it is 2.0. Protein, phenol, EtOH and other things often lower these ratios because they absorb at A280.

1. Dissolve the four similar pellets each in 25 ul 1 mM Na citrate Buffer/pH 6.4 or TE buffer (10 mM Tris, 1 mM EDTA pH8).

(Heat in 70oC block and vortex hard and repeatedly over 15 minutes.) Pool so that you have a 100 ul sample for each RNA prep.

{RNA STORAGE: Store at 4oC during sample use (this class). For storage over 1 week, can store at -80oC. For longer storage, add 3 volumes of ethanol and store at -80oC.}

2. Add 0.5 ml of DEPC-H2O to seven 1.5 ml tubes.

3. Label one "Blank" and the others (duplicates) after the samples: the two RNA samples and a positive control: 10mg/ml salmon sperm DNA.

4. Add 2 ul aliquots of samples to @ tube except "Blank".

5. Use micro UV-transparent disposable cuvettes and rubber or plastic transfer pipettes. Blank the machine to read 0 absorbance at A 260 nm with the "Blank" in a cuvette. Then repeat with dilute samples in cuvettes. Repeat the procedure for measuring the A280 of samples.

6. Estimate [RNA] (ug/ml) = A260* diln. Factor* 40

= A260 * 250 * 40

Therefore: [RNA] (mg/ml) = A260 * 10 (= ug/ul, too!)

7. Pipet 32 ug RNA into a clean 1.5 ml tube for each RNA prep. We want 32 ug aliquots of RNA for running replicate 8 ug samples on a Northern gel. If volume is less than or equal to 15 ul, store "as is" at -80oC. If volume is greater than 15 ul, precipitate the RNA by adding 3 volumes of 100% EtOH and 0.1 volumes of 3 M NaAc/pH 5.2. Vortex and store at -80oC.

8. To the rest of the RNA preps, add 3 vol 100% EtOH and store at -80oC. (This is a good way to store RNA without degradation for years.)

B. In vitro transcription - DO THIS FIRST!

NOTE : There are 3 common types of nucleotide probes: DNA oligonucleotides (ss), cDNA (ds) and cRNA (ss). For many applications, cRNA probes are superior over:

1. end-labelled oligonucleotide probes because they are:

a. longer (and therefore carry more label and have higher hybridization specificity)

b. uniformly labelled throughout (so they carry more label)

2. nick-translated or random-primed cDNA probes, because they only have the desired probe strand, not the other "sense" strand that increases background.

In addition, the binding of RNA:RNA hybrids is stronger than that of DNA:DNA hybrids.

REMEMBER:

1.the cDNAs are synthetic cloned fragments of the mRNAs

and

2. Knowing the information on the plasmid maps (See appendix) is critical to designing the probes (e.g. knowing what enzyme to linearize the plasmid with, which enzyme to transcribe with, and which strand (sense or antisense) is generated.

Circular plasmids must be linearized with a restriction enzyme to generate DNA templates suitable for in vitro transcription. You will use SP6 or T7 RNA polymerase to transcribe antisense cRNAs for actin and GAPDH mRNAs and 18S rRNA. Ambion's "pTRI-____" constructs are foolproof: already linearized, and have all the RNA polymerase sites on the side of the cDNA so as to make only antisense transcipts. (REMEMBER THAT RNA IS SINGLE-STRANDED!!!)

ANSWER THESE and all other PROTOCOL QUESTIONS IN YOUR NOTEBOOK

Q1.: If we wanted to probe a Northern blot with cRNA from poPR77A, which restriction enzyme and RNA polymerase would we use with that plasmid? (see map).

Q2.: If we wanted to do in vitro translation with cRNA from poPR77A, which restriction enzyme and RNA polymerase would we use?

[In vitro transcription kits can be obtained from various sources. Ambion's "Maxiscript" is OK and is the basis for the following reaction (one per group).]

A. In vitro Transcription using the Roche DIG-Labeling kit.

Each student should set up one in vitro transcription reaction for DIGOXIGENIN-labeling antisense 18S, GAPDH, or actin cRNA probes:

1. Thaw components at room temperature (RT) then store on ice.

EXCEPTION: RNasin and RNA Polymerase, like all enzymes, stay at -20oC always!

2. For sense cRNA add components from kit, in order, to a 0.5 ml tube at RT.

12 ul DEPC-H2O

2 ul 10X Transcription Buffer

1 ul RNasin

2ul 10 mM rATP, rCTP, rGTP, and 3.5 mM DIG-11-UTP

2 ul linearized DNA template

1 ul SP6 or T7 RNA Polymerase

3. Mix by pipetting up and down...gently! No bubbles.

4. Flash spin in a microfuge

5. Incubate 37oC for 1h

6. Add 1 ul RNase-free DNAse

7. Incubate 15 min at 37oC

8. Save 5 ul of each of the transcription reactions in 1.5 ml tubes for analysis on a urea 5% acrylamide short fat sequencing gel. Store these tubes and the original reaction tubes at -80oC.

9. For short fat acrylamide gels in next lab, wash 1 short and 1 long plate with soap and water and a 250 ml Erlenmeyer flask or beaker. Soak in 1 M NaOH overnight (O/N).

In Vitro Transcribed RNA (and RNase test) analysis on urea/acrylamide gel

A. Analyze in vitro transcription products on a 8M urea, 5% acrylamide gel called a "probe test gel or short, fat sequencing gel." These denaturing acrylamide gels are especially suitable for analysis of small (<400 base) DNAs and RNAs.

1. Make a probe test gel. (See Appendix recipe and plate setup for vertical gel.)

2. Add 15 ul of deionized formamide loading dye to your 5 ul cRNA aliquots as well as to your RNA samples from the first lab (RNase test). Similarly, prepare a sample of RNA Century (Ambion) markers. Leave the 15 ul aliquots of the cRNA reactions at -80oC!

3. Heat 68oC for 5 min.

4. Cool on ice.

5. Flush wells free of urea with 1X TBE in needle & syringe.

6. Load with elongated gel loading tips carefully! in the bottom of wells.

7. Run at 35 mamps for 1 h.*

8. Disassemble gel plates.

9. Stain the gel with ethidium bromide by soaking in 2 ug/ml EtBr for 5 min. Use gentle agitation.

10. Destain by soaking in d H2O for 5 min. with agitation.

11. Photograph the gel on a UV light box. Label the photograph completely (see Lab Rules A4) and tape it in your notebook.

12. Compare migration distances and brightnesses of bands with those in RNA marker lane. Note the migration of the tracking dyes, too (see table in APPENDIX) (Xylene cyanol is light blue & slow, while bromphenol blue is dark blue and fast).Mark the dye migration positions on the photograph so you could rerun the gel exactly if you wanted to.

13. For the next lab, treat a 250 ml Erlenmeyer flask or beaker and a mid-size horizontal gel apparatus with 1 M NaOH. Leave these to soak until next time.

14. Do calculations for Lab 5 step A6. Dilute a small amount of plasmid DNA template for in vitro transcription to 10 ng/ul.Store at -20oC.

*During the gel run, discuss DNA structure using human nucleotide models:(answer all questions in your notebook!)

a. Make a single-stranded six base random sequence and identify 5' and 3' ends. What types of bonds exist between the bases?

b. What is the chance that a specific 6 base sequence will occur randomly?

c. Reverse the sequence polarity. What bonds did you have to break?

d. Make a complementary strand of DNA. How are the strands oriented to each other? How is this strand's sequence related to the initial strand? What types of bonds are between the strands?

e. Make an EcoRI restriction enzyme site. What bonds does the enzyme cut? What makes the ends "sticky"? Do the ends have 5' or 3' overhangs?

Lab 5 10/4/02

Northern Gels (NorthernMax (Ambion) protocol) and Blotting

Be clean - protect apparati from Rnase contamination- clean diapers!

All DEPC reagents

A. Recover 32 ug total cellular RNA samples from the endometrial and liver extracts that you made (2 per student).

For each RNA prep, make a 32 ug sample to provide duplicate samples for two lanes. If samples from C7 of Lab 3 are precipitated start at 1 and continue. If not, start at step 6.

1. Spin 10 min at 4oC at 10,000X g

2. Discard supernatant by decanting

3. Dry sides of tubes with Kimwipe and remove residual ethanol from pellet with yellow tip if needed.

4. Air dry pellets 5 min.

5. Dissolve pellet in 15 ul 1 mM Na citrate at 68oC with vortexing.

6. If your sample was not precipitated, bring it up to 15 ul with 1 mM Na citrate. Add 45 ul Northern Sample Loading Dye and 6 ul of 0.1 mg/ml EtBr to each sample. These will be loaded in two lanes as described below. ALSO, prepare 10 ng samples of linearized plasmids with cDNAs for 18S rRNA, GAPDH, and actin (diluted to 10 ng/ul in Lab 4 step 14) in 5 ul 1 mM Na citrate + 15 ul dye. Add 2 ul of 0.1 mg/ml EtBr to each sample. Lastly, prepare a 15 ul sample of mouse liver control RNA to be split into two lanes during loading.

Q: What is the concentration of the control RNA? How much is in each lane?

7. Heat 68oC for 10 min.

8. Chill on ice

B. Prepare & Run gel. 2 people per gel: one student load the top row, one the bottom row of wells

START THIS FIRST OR SIMULTANEOUSLY WITH YOUR RNA SAMPLE PREPS (above)!

1. Rinse gel rig and beaker/flask well with house-distilled water. Melt agarose (0.8 g) in 72 ml DEPC-H2O in an RNASE-Free glass bottle or beaker. (Bring to a boil in microwave oven and mix by swirling: repeat 2 to 3 times).

2. Cool to 70oC.

3. In a fume hood, add 8 ml 10X Denaturing Gel buffer (formaldehyde and MOPS/pH 7.0, NaAc, and EDTA) and pour into RNase-free gel mold with the ends taped. Use two thin combs.

4. Load samples onto the gel under 1X Gel Running Buffer (dilute the 10X stock from the kit with your DEPC-H2O bottle, cover the gel with buffer to about 0.5 cm depth). LOADING ORDER[Skip spaces = "X"]

Millenium RNA markers, Positive control RNA , student1 Endometrial RNA, student1 Liver RNA, pTRI- actin, pTRI-GAPDH, pTRI-18S rRNA, student2 Endometrial RNA, student2 Liver RNA plasmid.

5. Run at 100 volts until dye front reaches bottom or sufficient separation occurs (1 to 1.5 h); can peek at gel progress with hand-held short wave UV lamp. Prepare materials for blotting during this time!!!!!!!!!!!

6. Take photo on UV box alongside a fluorescent ruler.

NOTE: MARK DYE POSITIONS ON GEL PHOTOGRAPHS. Label all photos completely! Name, date, identity!!! Number lanes on photo then desribe them in your lab notebook.

C. Northern transfer to nylon membrane

1. Cut wicks, blotting papers, and nylon membrane wet in transfer buffer as directed.

2. Soak gel in 10X SSC for 15 minutes with gentle agitation.

3. Assemble an upward capillary transfer as instructed in the diagram. You can use the rig you ran the gel in to do the transfer to nylon. Allow the transfer to continue with 500 ml of 10X SSC until the next lab.

Lab 6 10/11

Northern Blot hybridization

A. UV cross-link RNA to Northern blot

1. Remove all papers from the Northern transfer but keep the gel and blot together. , mark well positions on the blot with a sharp pencil or a black Sharpie marker. Also write initials and date. Put these marks on filter's back. Keep the blot RNA-side-up during subsequent handling.

3. Rinse filter for 30 sec. in 2X SSC with vigorous agitation.

4. Put blot on plastic wrap and on UV box. On the side of the blot, mark positions of 28S and 18S rRNAs and RNA markers. Also, mark places you will cut the blot in the future (to hybridize with different probes), e.g., on the blank lane #6. You can take a picture to confirm that the RNA transfer was good. Look at the gel on the UV box. Did all of the RNA transfer out?

5. Place wet blot on top of Whatman paper saturated with 2XSSC - all on top of plastic wrap.

6. UV crosslink RNA to nylon (use Stratalinker in energy mode: 120,000 (or 1,200,000 ?) ujoules).

7. Cut the blot into pieces to be probed with 18S rRNA and GAPDH and actin probes.

B. Prehybridization & Hybridization

Since the nylon membrane likes to bind things, background sites are blocked (bound) with non-specific DNA and protein. Usually, sheared salmon sperm DNA is used in prehybe to block these sites.

1. Warm the Ambion Ultrahybe hybridization solution to 65oC. Swirl to dissolve precipitates.

2. Make three plastic bags from a tube with the heat sealer. Double seals are a good idea. The bags should be 2 cm longer and wider than blots. Leave one end open and insert the dry blot . Wet it with 2X SSC. Pour 2X SSC out.

3. Add 5 to 10 ml Hybridization solution and seal bag. Note that bags are harder to seal when they contain the fluid, so you may have to turn up the sealing time on the heat sealer to get a good seal.

4. Incubate 68oC for 30 min. During this time, calculate how much of each probe to add to a fresh 10 mls of Ultrahybe to reach 10 to 20 ng/ml (about 0.1 nM).

5. Heat cRNA probe at 94oC for 10 min - chill on ice.

6. Cut off a corner of the bag.

7. Discard prehybe buffer. Add Ultrahybe containing the appropriate probe to each blot.

8. Reseal bag with heat sealer

9. Incubate 65oC overnight or over weekend.

10. Predict Blot results

Based on your blot photo and what you know about actin mRNA (2100 bases) and GAPDH mRNA (1400 bases), draw your expected hybridization results in your notebook. Use the 28S & 18S rRNA positions as markers. For the mouse, 28S rRNA is 4718 bases and 18S rRNA is 1847 bases. There should be 2 to 6 pg of GAPDH mRNA in 5 ug of the mouse liver RNA + control.

Q: How much RNA is in each rRNA band?

C. Assess quality of probes.

1. Make 4 serial 1:9 dilutions of each probe: Pipet l undiluted probe into a tube with 9 ul TE and mix. This is a 1:10 dilution. Repeat the dilution 3 times; so have 1:10, 1:100, 1:1000 and 1:10,000 dilutions.

2. On three 3 by 10 cm strips of nylon membrane, mark membrane with sharp pencil or black Sharpie pen "-1", "-2", "-3", "-4" at 2 cm intervals. Labelled one for each probe. Add initials and date.

3. Dot 1 ul of appropriate probe dilutions under labels. UV Cross-link RNA to membrane as you did for the Northern blot.

4. Air dry and store in a clean place. (You'll develop the dot blot along with the Northern blot in the next lab.)

Lab7 10/18

Northern Blot Washing and Development; Making cDNA by Reverse Transcription

A. Northern blot washing and development

After overnight hybridization, probe is maximally bound to specific sequences. It is also present on some non-specific sites. By reducing [salt], mainly in the form of SSC, hybridizations are tested for stringency. Usually temperature is increased as well so that probe-binding is specific for the target of interest.

KEEP BLOTS WET DURING THESE PROCEDURES or you'll generate a lot of artifacts.

1. Cut off corner of hybridization bag. Discard hybe solution.

2. Cut bag open and move blot to a clean tupperware container. Put all 3 blots in the same container. Seal the lids during washes so solutions don't spill onto the shakers!!!

3. Wash the 3 blots in 100 mls of 42oC 2x SSC with shaking for 15 min.

4. Discard wash and repeat.

5. Wash in 100 mls 0.5 X wash solution [Maleic acid buffer (1X = 0.1 M maleic acid, 0.15 M NaCl, pH 7.5) with 0.3% Tween-20] at 65oC for 15 min. Repeat once.

6. Use CDP-Star detection system at RT:

a. Add in the probe dot blot from Lab 6C at this step. Equilibrate membranes in washing buffer 1 min:

b. Allow chemiluminescent substrate to come to RT.

c. In freshly washed tupperware, block the membranes for 30 min in 20 ml block solution (1% (w/v) blocking reagent in maleic acid buffer without Tween). During gentle shaking, membranes should move independently from each other.

d. Discard block and incubate in Anti-DIG-Alkaline Phosphatase antibody solution (1:20,000 diluted in block solution) for 30 min. Use a minimal volume (20 ml) in a small clean container like a yellow tip box lid. During gentle shaking, membranes should move independently from each other.

e. Discard the antibody solution and wash twice in washing buffer for 15 min each time.

f. Discard wash and equilibrate membrane in detection buffer for 2 min.

g. Pipet 1 ml of CDP-star detection reagent diluted 1:100 in detection buffer onto each blot and cover with plastic wrap. Incubate 5 min, then pour off excess reagent.

7. Wrap blot in saran wrap. To keep the blot wet and the film dry, double fold the plastic wrap and tuck all edges under the blot. As always, RNA side up!

8. Place in cassette.

9. Go to the dark room and lay a piece of film on the blot. Bend the lower right corner of the film. Close the cassette. Make sure you fold the flaps so it is light tight!

10. Place cassette at 37oC or room temperature. Develop the film in 30 min. If desired, place new film on and expose longer...O/N?.

B. Making cDNA by Reverse transcription

To produce cDNA for cloning, mRNA is reverse transcribed. Typically, an oligo dT primer is annealed to the poly A tails of the mRNA's. The Reverse Transcriptase (RT), the enzyme retroviruses use to copy their RNA genomes, copies the RNA into cDNA. This reaction is used for first strand cDNA synthesis, the first step in formation of all cDNA libraries, and is the basis for true molecular cloning.

Each person will perform one RT synthesis of cDNA on their favorite RNA sample.

1. Spin down stock RNA sample after adding 1/10 vol 3M NaAc and mixing.

Dissolve in a volume of 1 mM Na citrate to make solution 1 ug/ul.

(estimate from absorbance measurements). Measure A260. Pipet 10 ug into clean tube and bring up to 25 ul volume with 1 mM Na citrate.

2. Combine in a 1.5 ml tube at RT, in order:

5.0 ul DEPC-H2O

5 ul 5X AMV RT Buffer

1 ul 100 mM DTT

2 ul dT17 primer

5 ul RNA (2 ug)

3. Heat 68oC 5 min.

4. Let cool to RT slowly.

5. Add 5.0 ul 5 mM dNTP

1 ul RNasin

1 ul AMV RT

6. Incubate 37oC, 1 h.

7. Heat at 94oC for 5 min. to kill RT.

8. Store at -20oC.

Lab 8 10/25

Analysis of Blots; Quantitative RT-PCR

A. Northern Blot Analysis

1. Develop the autoradiograph in the film processor. Label films with exposure date, time and index to your notebook. Align with the blot and mark the well, 28S & 18S rRNA positions on the film. What are the positions of the hybridized bands? Are the bands more intense in RNA samples from one tissue compared to the other?

2. Qualitatively assess the blot results:

a. Is exposure optimal?

b. How many bands are evident? Are they in the expected tissues?

c. What is the size of the darkest band? To do this measure migration distances for the band on the Xray film, and for 28S (4800 bases) and 18S (1800 bases) rRNAs on the EtBr stained gel photo using UV ruler as a guide. On graph paper, plot log base length against migration distance for the rRNAs. Find log bases from the plot, using the migration distance of the band of interest. Find antilog to get number of bases. If RNA markers show up, you can use those instead of or in addition to the 18 and 28S rRNAs.

3. Quantitate blots –

a. Can use densitometry on the Xray film. Need a good scanner and analytical software such as BioImage IQ.

b. Many machines are being developed for direct scanning of blots. These are very powerful because they avoid the limitations of film.

B. Quantitative PCR analysis of ER mRNA in an RNA sample

NOTE:Be clean (Don't introduce exogenenous DNA) USE AEROSOL BARRIER TIPS! WEAR GLOVES!

PCR amplification is very useful in detecting small amounts of nucleic acids. Since the amplification can be described mathematically in theory, then it should be possible to calculate back to the original concentration of template. Problems arise because the exponential amplification is seldom realized. To reduce variability and gain quantitative ability, PCR reactions are done with the fewest possible cycles under limiting reagent conditions. An internal competitive template, with the same primer/sites on a shorter piece of DNA, can be added in increasing amounts to a PCR reaction. In the reaction with equivalent products: large from the template of interest and small from the internal competitor; the starting amounts of template are equivalent. So by this PCR titration with competitor template and gel analysis with EtBr staining, templates of interest can be quantitated. Quantitative PCR is very well explained in Clontech's MIMIC manual.

How do you get template DNA? The quantitative PCR can be tested on linearized plasmid DNA. But to use PCR to estimate [specific mRNA] in an RNA sample, it is first reverse transcribed to cDNA as you did in the last lab.

Each person will do a set of five quantitative PCR reactions on one RT-cDNA sample, as well as (+) and (-) PCR controls.

1. At RT, combine (in order) 8X amounts of the first five reagents to make a master mix. Add 23 ul of master mix to seven 200 ul thin-walled tubes, then add the template and internal control DNAs to each individual tube.

Master mix: 17.5 ul sterile H2O X8 = 140 ul

2.5 ul 10X Taq Buffer containing Mg++ X8 =20ul

1 ul 5 mM dNTPs X8 = 8 ul

1 ul primer A (ERPCR1, 10 ng/ul) X8 = 8 ul

1 ul primer B (ERPCR2,10 ng/ul) X8 = 8 ul

2. Do serial dilutions (10E–1, 10E-2, 10E-3, 10E-4) in sterile water of the provided internal control DNA(linearized poER8short, 100 amol/ul). Add DNAs to your tubes:

TUBE #1 1 ul RT-cDNA and 1 ul 10E-4 internal control

TUBE #2 1 ul RT-cDNA and 1 ul 10E-3 internal control

TUBE #3 1 ul RT-cDNA and 1 ul 10E-2 internal control

TUBE #4 1 ul RT-cDNA and 1 ul 10E-1 internal control

TUBE #5 1 ul RT-cDNA and 1 ul undiluted internal control

TUBE #6 1 ul positive control plasmid (poER8, linearized, 1 amol/ul) and 1 ul H2O

TUBE #7 2 ul H2O [negative (no template) control]

All should have 25 ul final reaction volumes.

Note: PCR primer sequences are:

ERPCR1 - (5') agcccagcggctacaggtgc

ERPCR2 - (5') gcaggcctggcagctcttcctcct

3. Program the PCR machine to do 40 cycles of:

94oC for 30 sec: strand separation

50oC for 30 sec: primer annealing

72oC for 1 min: polymerization

4. Put your 7 PCR tubes in the PCR machine. Add 0.25 ul Taq DNA Polymerase with a P-2 Pipetteman and tips during a 94oC hot start (make machine hold at 95oC for 2 min then add enzyme. You can also do this in a hot block or bath. This "hot start" prevents lots of nonspecific product from primers annealing non-specifically with the template at RT.) Run the cycling program after enzyme addition.

5. Make one 2% agarose gel for each two students:125 mls of 2% agarose gel + 0.5 ug/ml EtBr in 1X TAE; use 2 thin combs for each gel .

6.. Store PCR reactions at -20oC. Store gel in the gel mold with the comb, wrapped in saran wrap with a little buffer and in a sealed tupperware container or pyrex dish at 4oC.

Lab 9 11/1

Quantitative RT-PCR Analysis; PCR Subcloning

A. Quantitative PCR Analysis

1. Add 2.5 ul 10x DNA dye to each 25 ul of PCR reaction. Mix by pipetting up and down.

2. Load the entire samples (or as much as possible in the well) on the 2% gel made in previous lab. Also load pGEM DNA markers (2ug = 20 ul).

3. Run at 120 V for 1 h.

4. Photograph.

5. Estimate

a. Sizes of products in bp.

b. [target cDNA] in RT-cDNA reaction.

NOTE: The ratio of the target cDNA (315 bp) to the internal control (249 bp) is the critical endpoint. Find the reaction where the two bands are nearest to being equivalent in brightness of EtBr staining, and that is the reaction that started out with equal amounts of target mRNA and internal control. Since you know how much of the latter you added, you know how much of the target was in the sample. You can now determine the concentration of mRNA in your original RNA prep.(You can assume the RT reaction made one cDNA copy of each mRNA present.)

To make this technique amenable to comparing mRNA levels in a large sample set, a single internal standard concentration equal to the average target concentration is used with each sample. Radioactive dNTP's can be used to label the products so their ratios can be quantitated. There are machines that quantitate UV fluorescence directly from gels, too.

NOTES on INTERPRETING AGAROSE GEL INFORMATION:

By comparing the bands in plasmid lanes with DNA standards, one can describe

a. The molecular size of the fragment, and

b. The [DNA] of each fragment

For (a), compare migration distance to that of DNA standard fragments - estimate bp size.

For (b), compare brightness of bands to those of DNA fragments - estimate ng. Divide by the amount of sample loaded to get [DNA]. This is often more reliable quantitation than A260

measures!!!

NOTE: MARK DYE POSITIONS ON GEL PHOTOGRAPHS. Label all photos completely! Name, date, identity!!!

B. PCR sub-cloning (see cartoon following)

One of the most useful applications of the polymerase chain reaction (PCR) is easy subcloning. Subcloning, taking a piece of cloned DNA and transferring to a new vector, used to be limited due to the scarcity of naturally occurring restriction enzyme sites. One was forced to choose DNAs with restriction sites, often these were for weird, expensive, and inefficient restriction enzymes. With PCR, restriction enzyme sites can be engineered on any DNA by adding the desired sites to the ends of the primers. Note that 5' and 3' primers must have different sites to prevent them from annealing. After restricting the PCR product, sites are different on the ends and can only enter a similarly restricted vector in one orientation: therefore, this is "Directional cloning."

Although PCR can be temperamental, subcloning fragments less than 1000 bp is, dare I say, fairly easy.

Here, we'll subclone our beta globin cDNA into a different plasmid vector. The old vector was pBluescript (Stratagene); the new pET-5a (Promega).

Q: What specific advantages does the new vector have (see Promega catalog or protocol book)? List these in your notebook.

Do 1 PCR reaction per person. Be clean (Don't introduce exogenenous DNA): USE AEROSOL BARRIER TIPS! WEAR GLOVES!

1. Combine, in order, at RT in a 200 ul thin-walled PCR tube

17.5 ul H2O

2.5 ul 10X Taq Buffer containing MgCL2

1 ul 5 mM dNTPs

1 ul primer Xba-GLOB (0.25 ug/ul)

1 ul primer GLOB-R1 (0.25 ug/ul)

1 ul linearized pGLOBcdsonly

0.5 ul Taq DNA Polymerase

Note: primer sequences are:

Xba-GLOB (5') gctctagatgctggttgtctacccatgg

GLOB-R1 (5') gcgaattctgaagttctcaggatccacg

2. Mix by pipetting up and down

3. Program a PCR machine for 35 cycles of:

94oC for 30 sec: strand separation

50oC for 30 sec: primer annealing

72oC for 1 min: polymerization

4. When the PCR is complete, the reactions will be stored at –20oC until the next lab session.

Lab 10 11/8

DNA Restriction and Ligation

A. DNA Restriction and purification on gel

Ligations are the weak point in most cloning procedures. Here we are set for success by using "sticky ends" restriction sites with 4 base overhangs that like to pair up. We'll also do the ligation directly in low melting agarose to minimize loss of DNA during purification.

NOTE: Restriction Enzyme cuts usually contain 1-2 ug DNA and 5-10 units of enzyme in 20 ul. Working buffer strength is always 1X. Use the buffer supplied with the enzyme if it is a single enzyme cut. If using two enzymes (as here), use a buffer compatible to both! Which one should you use????? There is a table in the Promega book that will help you choose.

1. Add 25 ul of TE to the PCR reaction and transfer the 50 ul to a 500 ul microfuge tube.

2. Extract with Phenol/Chloroform/IAA (25:24:1) pH 8.(This means to add an equal volume of Phenol/Chloroform/IAA, vortex to make an emulsion, microfuge 2 min., then transfer the upper aqueous phase that contains the DNA to a clean, labeled tube.)

3. Extract with Chloroform/IAA (24:1)

4. Set up a restriction digest for the PCR product:

16 ul PCR product (insert)

2 ul 10X ?????? Buffer

1ul EcoR1

1 ul Xba I

20 ul TOTAL volume

5. AT THE SAME TIME, RESTRICT 0.5 ug of the vector pET-5a (see map in Promega book) as in 4. Check calculations for the reaction with the instructor.

6. Incubate at 37oC for 1 h.

7. Make a 0.8% low melt agarose gel + EtBr. Raise comb by adding small squares of tape. These gels are like soft jello - Hard to handle!! Be careful removing combs, etc.

8. Add 2 ul 10X Loading dye to the digest reaction. Load it and 1 ug Lambda HindIII EcoR I DNA markers on the gel. Run at 100 V for 30 min.

9. Cut out bands and remove excess agarose while visualizing under Long wave UV light. Wear goggles!

B. DNA LIGATION

1. Melt DNA + agarose 70oC, 10 min. Then keep tube at 37oC until through pipetting.

2. Label two ligation tubes. Put them at 37oC. Add 1 ul vector DNA in gel to each. To the ligation tube, add 3 ul insert DNA in gel and 5 ul H2O. To the control tube add 8 ul H2O. Keep these at 37oC for at least 2-3 min and until the master mix is added.

3. Make a Master mix composed of:

2 ul 10X Ligase Buffer x 3 = 6 ul

8 ul H2O x 3 = 24 ul

1 ul T4DNA Ligase x3 = 3 ul

11 ul per reaction

Add 11 ul ice-cold mix to each ligation tube. Finger flick the tube immediately and slam on ice. Reactions will gel while ligation occurs.

4. Incubate 15oC, O/N. This is done in a hot block in a 4oC room.

Lab 11 11/15

Transformation of Bacteria

"Bacterial transformation" relates to the change of bacterial phenotype by introducing a

plasmid containing an antibiotic resistance gene. "Competent cells" are made receptive to plasmids by making their membranes permeable with calcium treatment. Plasmids adhere to cells, enter on heat shock, and cells are selected for antibiotic resistance on plates after a 1 h recovery period in broth. NOTE: All waste contaminated with E. coli must be killed with bleach or discarded in "BIOHAZARD BAGS," which are autoclaved prior to disposal.

Each group does three transformations: one from the vector only control ligation and one from the ligation that has DNA insert too, as well as a positive control for transformation: 10 ng of circular plasmid. For the last, use any plasmid.

1. Thaw cells on ice (20 min.)

2. Pipet 100 ul into a cool 1.5 ml tubes: 1 for each ligation (vector only and vector+insert), 1 for the positive control

3. Melt ligations at 70oC, 10 min.

4. Cool ligations to 37oC in block, at least 2 min.

5. Add 1 ul ligation to cells; mix by pipetting up and down.

6. Incubate on ice 30 min. Mix every 10 min. by tapping th tube gently.

7. Heat shock 42oC, 45 sec (Be exact here!). No shaking.

8. Ice for 2 min.

9. Add 400 ul room temperature S.O.C. broth.

10. Agitate cultures gently, 37oC, 1 h. Tube turners or rockers are good for this.

11. Spread all 500 ul on a LB + Ampicillin plate for the transformations from ligations. Spread only 50 ul for the transformation of circular plasmid.

12. Incubate 37oC O/N. Invert plates if all the liquid goes into the plate. Incubate wet plates right side up.

Thursday 11/21 (any time between 2 to 3 p.m.?)

1. Count colonies on the ligation plates. Compare to the number of colonies on vector only control plate. If you have twice as many colonies in the ligation transformation plate, then 50% should contain the insert!

2. Make 4 - 10 ml overnight cultures of LB + Amp in 50 ml tubes. Label tubes 1-4 (initials and date).

3. Inoculate @ with 1 colony: from vector only plate (1 culture) or from ligation plate (3 cultures).

4. Incubate with strong agitation (200 rpm) at 37oC O/N.

Lab 12 11/22

Plasmid DNA Miniprep

NOTE 1: Phenol for DNA is buffered with Tris to pH8 for optimal partitioning. For RNA, Phenol is water-saturated and is ~pH5. Use the correct phenol for your nucleic acid!!!

NOTE 2: ALSO, Phenol is corrosive - causes burns! Be careful! Wear safety glasses!!!

NOTE 3: CHCl3 (Chloroform) dissolves things like styrofoam and polystyrene - use glass graduated pipets and polypropylene 15 & 50 ml tubes.

In a short, mind-numbing period of tube shuffling, one can extract plasmid DNA for analysis of desirable clones

1. Spin down 1.5 ml overnight culture cells in a 1.5 ml tube - (DISCARD all materials from steps 1 -3 that are contaminated with cells in BIOHAZARD BAGS.)

2. Save the rest of the overnight culture at 4oC - IF it is a good clone, you'll want to make a glycerol stock for long term storage and a streak plate for short term use.

3. Remove the medium by aspiration, leaving the bacterial pellet as dry as possible.

4. Resuspend the pellet by trituration (pipetting up and down) in 150 ul of an ice-cold solution of:

50 mM glucose

10 mM EDTA

25 mM Tris/ HCl (pH 8.0)

Add 4 mg/ml lysozyme (added freshly to the solution)

5. Store for 5 minutes at room temperature. The top of the tube need not be closed during this period.

6. Add 300 ul of a room temperature solution of: 0.2 N NaOH + 1% SDS

Close the top of the tube and mix the contents by inverting the tube rapidly two or three times. Do not vortex. Store the tube on ice for 5 minutes.

7. Add 225 ul of an ice-cold solution of potassium acetate (~pH 4.8). [This reagent was made

up as follows: To 60 ml of 5 M potassium acetate, add 11.5 ml of glacial acetic acid and 28.5 ml of H2O. The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.]

Close the cap of the tube and vortex hard, put on ice, vortex again. Store on ice for 5 minutes.

8. Centrifuge for 15 minutes in an micro-centrifuge at 20oC.

9. Transfer 600 ul supernatant to a fresh tube. (Avoid all white, solid garbage).

10. Add an equal volume of phenol/chloroform pH8. Mix by vortexing. After centrifuging for 2 minutes in an Eppendorf centrifuge, transfer the aqueous phase to a fresh tube.

11. Add two volumes of ethanol at room temperature. Mix by vortexing. Stand at room temperature for 2 minutes.

12. Centrifuge for 5 minutes in an Eppendorf centrifuge at room temperature.

13. Remove the supernatant. Stand the tube in an inverted position on a paper towel to allow all of the fluid to drain away.

14. Add 1 ml of 70% ethanol. Vortex briefly and then centrifuge.

15. Again remove all of the supernatant. Air dry the pellet briefly (5 minutes) after wiping away residual ethanol with a Kimwipe.

16. Add 50 ul of TE (pH 8.0). Vortex and incubate at 37oC to solubilize DNA (about 5 minutes).

17. Store at -20oC until next lab session.

Lab 13 12/6

Miniprep plasmid DNA Restriction and Gel Analysis

A. To analyze the four plasmid preps from overnight cultures, we'll restrict each with EcoRI and Bam HI to see if the 400bp insert is present.

1. We will cut inserts out of the vectors for each plasmid prep to verify it. Best pipetting technique minimizes pipetting steps (and, thus, work and error). So, to set up a set of 20 ul restriction digestions, make a master mix of common components:

In single reaction x5 = in master mix

6ul H2O x5 =30 ul

2 ul 10X????? buffer x5 =10ul

1ul 1mg/ml RNase x5 =5 ul 1mg/ml RNase

0.5 ul Bam H1 (10 U/ul) x5 = 2.5 ul Bam H1

0.5 ul EcoR1 (10U/ul) x5 = 2.5 ul EcoR1

Use 10ul/rxn

Mix gently. Pipet 10ul into 4 tubes.

Add 10 ul of individual plasmid preps, one to each tube. Incubate 1 h at 37oC. ALSO Prepare one uncut plasmid sample: 10 ul plasmid + 10 ul TE and incubate at 37oC. After incubations, add 2 ul 10x DNA dye to each of the five tubes. Store leftover plasmid at -20oC.

2. Make a 1% agarose gel + 0.5 ug/ml EtBr in 1X TAE. Use a mid-size apparatus and two 14-well combs.

3. Add 2 ul 10X DNA dye to @ digest.

4. Load gel with uncut plasmid (1 sample) and 4 plasmid digests. Also load Lambda Hind III EcoR1 markers (1 ug) in one well of top and bottom halves of each gel.

5. Run at 120V, 1 h.

6. Photograph under UV light.

7. By comparing the bands in plasmid lanes with Lambda standards, one can describe

a. The molecular size of the fragment

b. The [DNA] of each fragment, and

c. the molecular form (circular vs. linear) of the fragments

For (a), compare migration distance to that of Lambda standard fragments - estimate bp size.

For (b), compare brightness of bands to those of Lambda fragments - estimate ng. Divide by 10 ul (amount of sample loaded) to get [DNA]. This is often more reliable quantitation than A260 measures!!!

For (c), note that uncut plasmids run fast as two forms, supercoiled and nicked circular. The restricted plasmid from vector only transformation shows that linear DNA is slower. Since markers are linear DNAs, their migration only relates to other linear DNAs.

NOTE: MARK DYE POSITIONS ON GEL PHOTOGRAPHS. Label all photos completely! Name, date, identity!!!

Lab Clean-up:

Biohazard waste

Discard phenol in appropriate bottles

Samples - save?

Return: 10 ml pipettors

APPENDIX

Use of Micropipettors

1. Choose the correct pipet. For volumes:

1-20 ul P20

20-200 ul P200

200 - 1000 ul P1000

2. Set the desired volume by holding the pipetman in one hand and turning the volume adjustment knob until the correct volume shows on the indicator. For best precision, always approach the desired volume by dialing downward (at least one-third revolution) from a larger volume setting.

3. Attach a new tip to the shaft of the pipet. Press tip on firmly to ensure airtight seal. Chose the correct tip.

P20 yellow tip

P200 yellow tip

P1000 blue tip

4. Depress plunger to first positive stop. Hold pipetman vertically and immerse disposable tip into sample liquid 2mm.

5. Allow the push button to return slowly to the up position. Never permit it to snap up.

6. Wait 1 or 2 seconds to ensure that the full volume of the sample is drawn into the tip.

7. Withdraw tip from the sample liquid. Wipe the sides of the tip on the sides of tube to remove any remaining liquid.

8. To dispense the sample, place the tip end against the side wall of the receiving vessel and depress the plunger slowly to the first stop. Then depress the plunger to the second stop to expel any residual liquid in the tip.

9. With the plunger fully depressed, withdraw pipetman from the vessel. Then allow the plunger to return to the top position.

10. Discard tip by depressing the tip ejector button. A fresh tip should be used for each sample.

Terribly Difficult Calculations

1. Molar solutions

1 M (mole per liter) means the solution has 1 molecular weight mass (g) per volume (liter) of soln.

A mole is a number of molecules:

6.022 x 10E23, Avogadro's number

To make 500 mls of 0.5 M NaCl (NaCl is58.55 g/mole) you need (0.5 liters)

(0.5 mole)= 0.25 mole

liter)

0.25 mole * 58.55 g/mole = 14.6 g

So: Add 14.6 g NaCl power and bring final volume to 500 ml with H_2O.

2. We typically work with concentrated stock solutions. For example, our Tris/acetate/EDTA (TAE) is made as a 50X stock. We run gels in 1000 mls of 1X TAE. The way I do DILUTION PROBLEMS is:

[Stock] * y = [Desired] * Desired volume; where y is the volume of stock. To find Y needed to make 1 li of 1X TAE from a 50X stock:

50X * y = 1X * 1000 ml

y = 1X/50X * 1000 ml = 20 ml

So add 20 ml 50X TAE to a 1 liter graduated cylinder. Bring volume to 1 li w/ dH2O.

3. Note: Dilutions are applicable to problems of pipetting very small amounts. If you want to add 0.2 ul, dilute the material 1:9 and pipet 2 ul with a P-20.

4. Percentage solutions should have a (v/v) or (w/v) or (w/w) following.

a. (v/v) relates volume to volume, indicating both components are liquids: e.g. 100 mls of 75% (v/v) EtOH is made with 75 mls EtOH + 25 ml H2O

b. (w/v) indicates solid to liquid ratio: e.g., 10 mls of 10% (w/v) ammonium persulfate (APS) is made w/ 1 g of APS to 10 ml final volume with water.

c. (w/w) is rare, indicating a weight to weight relationship. To make 10 mls of a 10% (w/w) APS soln, you could weigh 1 g APS on a scale and then add water until solution weight is 10 g. (That would be 9 g = 9 mls since density of H2O is 1 g/ml).

5. Of course, these calculations can be combined. For example, to make 500 mls of 0.5 M NaCl in 1X TAE,

Combine 14.6 g NaCl with 10 mls 50X TAE. Bring volume to 500 mls with H2O.

Easy!

PROBE TEST GEL

(short, fat sequencing gel)

5% acrylamide/urea gel

25.5g urea

19.5ml H2O

12ml 5X TBE

7.5ml 40% (w/v) acrylamide (19:1 acryl:bis)

60ml final vol.

Heat to 37oC to dissolve urea

Cool to below RT

Filter (~optional)

Add 400 ul 10% APS (less than 1 week old, make 1ml)

50 ul TEMED

Clean gel plates with soap, rinse with H2O extensively, then wipe with EtOH and Kimwipes. Set up plates as in Fig. 2 (except don't clamp over sponge).

Pour into 1.5mm thick vertical gel slab. Add comb.

Bubbles = Bad

Should polymerize in 15 min.

Rinse wells with 1X TBE immediately after pulling the comb and just prior to loading.

Samples in 80% (v/v) formamide loading dye

Heat 68oC 5 min for RNA. For DNA, 94oC 5 min.

Run at 25 to 35 mamps.

LIGATION IN LOW-MELT AGAROSE

1. Run restricted vector and insert DNAs on 0.8% low-melt agarose gel in TAE buffer. (If vector is cut with a single enzyme, treat with call intestinal phosphatase (CIP) prior to gel run to prevent recircularization of the vector). Cut out bands and remove excess agarose while visualizing EtBr-staining under long wave UV light.

2. Melt DNA + agarose 70oC, 10 min.

3. For @ ligation reaction, combine H2O and DNA + agarose in 9 ul volume (usually 1 ul vector and 3 to 8 ul insert). Put at 37oC for 2-3 min. Perform a vector ligation control reaction without insert.

4. Add 11 ul ice-cold ligase mix composed of

2 ul 10X Ligase Buffer

1 ul Ligase

7 ul H2O

1 ul 10mm ATP

for each reaction. Flick tube immediately! Immediately slam on ice. Reactions will gel while ligation occurs.

5. Incubate 15oC, O/N.

6. Melt ligation 70oC, 10 min. Transform with 1 ul.

Dye Migration Related to bases in a denaturing gel (d) or base pairs in a non-denaturing gel (n):

Acrylamide % Bromphenol Blue Xylene Cyanol

3.5% n 100 -

5% n 65 -

5% d 35 130

6% d 26 106

8% n 45 -

8% d 19 75

10% d 12 55

12% n 20 -

20% n 12 -

e.g., in a 5% acylamide + urea gel (denaturing), Bromphenol Blue comigrates with 35 base nucleic acids.

Agarose Gels for DNA

1% 1.5%

1g Agarose 1.5g Agarose

100ml 1X TAE 100ml 1X TAE

10ul 10mg/ml EtBr 10 ul 10mg/ml EtBr

Mix in 250ml beaker, cover with Saran Wrap, heat in microwave until solution boils 3 times. Allow to cool to 60oC, pour gel.

First Named Inventor	:	Michael E. Spurlock	
Appln. No.	:	09/928,522	
Filed	:	August 13, 2001	Group Art Unit: 1647
Title	:	Bovine Leptin Protein, Antisense and Antibody	Examiner: C. J. Saoud
Docket No.	:	LL31.12-0015	

EXHIBIT C

of

DECLARATION

submitted under 37 C.F.R. 1.132

Neumaier, M., Braun, A., and Wagener, C., Fundamentals of Quality Assessment of Molecular Amplification Methods in Clinical Diagnostics, Clinical Chemistry, 44:12-26 (1998)

Clinical Chemistry 44: 12-26, 1998;
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Special Report

Fundamentals of quality assessment of molecular amplification methods in clinical diagnostics

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Abstract

The increasing interest in molecular biology diagnostics is a result of the tremendous gain of scientific knowledge in genetics, made possible especially since the introduction of amplification techniques. High expectations have been placed on genetic testing, and the number of laboratories now using the relevant technology is rapidly increasing—resulting in an obvious need for standardization and definition of laboratory organization. This communication is an effort towards that end. We address aspects that should be considered when structuring a new molecular diagnostic laboratory, and we discuss individual preanalytical and analytical procedures, from sampling to evaluation of assay results. In addition, different means of controlling contamination are discussed. Because the methodology is in constant change, no general standards can be defined. Accordingly, this publication is intended to serve as a recommendation for good laboratory practice and internal quality control and as a guide to

troubleshooting, primarily in amplification techniques.

Introduction

The following recommendations on quality assessment of molecular biology methods in clinical diagnostics refer to the preanalytical and analytical steps, particularly those of amplification techniques. In particular, the polymerase chain reaction (PCR) and methods based thereon are those primarily used for developing laboratory tests that have potential for future routine applications. We have not attempted to deal with individual applications, which are at present in constant change and evolution and for many of which the clinical significance has yet to be established.

Because of the special requirements associated with amplification techniques, the aspects of good laboratory practice dealt with in depth are those regarding preanalytical and analytical aspects of nucleic acids amplifications. Many of the recommendations are aimed at avoiding contaminations and facilitating timely recognition of contaminants, should they occur. Although every amplification assay is prone to contamination, the technical effort associated with different types of nucleic analyses varies widely. For example, genotyping patients usually does not require optimization of the amplification conditions for improved detection limits. On the other hand, for detection of minimal residual disease or for virus detection, a very low detection limit is prerequisite. Accordingly, contamination risk may be not a major problem in one application but obviously can be critical in another. Also, if different enzymes have to be used in subsequent steps of a given test, as in reverse transcriptase (RT) PCR, the handling of the additionally required material must be considered a potential contamination hazard.

The potential of contamination is especially serious if, within the scope of diagnosis, the same DNA sequence is amplified repeatedly, or if amplification products are subjected to additional rounds of amplification, as exemplified in so-called seminested or nested PCR procedures. In nested PCR, the specificity of amplification can be enhanced through use of a second set of internal primers. As in "one-step" PCR, an excessive number of amplification cycles will generate nonspecific signals. In addition, nested assays are particularly prone to contamination because the PCR products generated during the first round of amplification are usually pipetted into new reaction tubes before reamplification. Alternatively, execution of nested PCR in a single reaction vessel, where possible, is preferable to the usual nested PCR approach (1)(2). In general, the gain in specificity by reamplification of the products of the first round should be weighed against the increased risk of contamination. At present, the use of nested PCR application cannot be generally recommended for clinical diagnostics but should be performed only by experienced laboratories.

Regardless of the amplification system being considered, the possibility of spreading the amplification products by aerosols into other reaction vessels as a source of contamination always has to be a general concern. Avoidance of contaminations requires careful planning of preanalytical and analytical steps. For the purpose of these fundamental recommendations, we presume that a molecular diagnostic

laboratory using amplification methods will provide a test program with assays that differ in their detection limit requirements, e.g., HLA typing and virus detection in blood specimens. The precautions taken should always reflect the needs of the most critical assay and should aim at the highest quality with respect to the test program. The laboratory staff must be clearly aware of the consequences of inadequate performance and quality control, and appropriate training must be given within the laboratory to ensure the high skills needed for molecular analysis. Once a contamination of reagents or stock solutions has occurred, it is often very difficult and time-consuming to localize and eliminate its source; consequently, all of the reagents may have to be discarded and replaced. Therefore, the aspects of laboratory organization and work flow discussed here are intended to assist the staff in preventing contaminants and to eliminate potential sources thereof. However, because danger of contamination cannot be entirely disregarded, even in the best circumstances, appropriate internal quality control is absolutely essential.

The current recommendations aim to define good laboratory practice rules for molecular biological methods. For the reasons given above, we do not differentiate between the more error-prone methods and those that are less so. We particularly emphasize techniques that utilize PCR, the most widely used method in the emerging field of nucleic acids diagnostics. However, most of the aspects that apply to PCR are also valid for other target amplification techniques.

1. Laboratory Organization and Laboratory Equipment

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- ▲ Introduction
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- ▼ 2. Preanalytical Aspects
- ▼ 3. Factors Interfering with...
- ▼ 4. Quality Controls
- ▼ References

The problems associated with the avoidance of contamination in PCR necessitate a decisive and strictly- adhered-to laboratory organization, including room and space planning. Ideally, a PCR laboratory should be divided into four separate work areas, each having dedicated special equipment for: *(a)* reagent storage and set-up, *(b)* sample preparation, *(c)* PCR reaction mix assembly and amplification, and *(d)* PCR product analysis. The following guidelines refer to these specific work areas.

The respective rooms must be marked as related to the specific areas; on no account may equipment such as pipettors or reagents be removed from their respective work area or exchanged between work areas. Access to the individual work areas should follow a strict sequence, i.e., proceeding in only one direction, from the reagent storage and set-up area to the PCR product analysis area (access sequence). Previous experience has proven that ignorance, thoughtlessness, or carelessness in adhering to the said sequence can lead to the spreading of severe carryover contamination. Consequently, use of different laboratory coats (e.g., of different colors) is recommended in the individual work areas to identify directly a potential source of contamination. In addition, the space-specific coats must be left behind when workers leave the respective work area.

The procedures and the access sequence must be adhered to by all staff accessing the laboratory area, including the cleaning personnel, for improper cleaning procedures can be a major source of contaminants. In principle, the laboratory is cleaned from reagent storage and set-up in the direction of

the PCR product analysis area. Likewise, separate cleaning utensils should be available for each work area to prevent cross-contamination.

1.1. reagent storage and set-up

1.1.1. Operations.

The following operations are done in the reagent storage and set-up area: preparation of stock solutions, preparation of aliquoted solutions, and preparation of master mix solutions. Cleaning of the workplace has to be performed immediately after the termination of the work. Also, specific work areas must not be accessed if work was performed earlier in any of the other working areas, particularly in the PCR product analysis area.

1.1.2. Job description, work flow.

Delivery of stock reagents and material for sample preparation is best done directly to the reagent storage and set-up area. Delivery to the PCR analysis area should be avoided (see below). Vessels containing reaction mixtures should always be centrifuged briefly before opening. The required reagents are stored exclusively in this area and processed here into the stock solutions needed.

After the stock solutions have been checked for suitability, they should be divided into aliquots for storage and further use, to reduce the danger of contamination through frequent opening of reaction vessels and pipetting.

Vessels containing reaction mixtures should always be centrifuged briefly before freezing. In general, most solutions used for PCR are stored frozen. Frequent use of freeze/thaw cycles of master stock solutions to remove aliquots for individual reactions must be avoided; instead, freeze stock solutions in small aliquots. Because the required volume for these stock solutions is determined by the number of PCR reactions usually carried out in one assay run in the laboratory, no specific volume recommendations are given here. Aliquot stocks of suitable sizes are predispensed into microreaction vessels and are subsequently frozen for storage. Plastic containers such as Tupperware(TM) are ideal for keeping batches of frozen stocks.

In addition, the reagent storage and set-up working area can serve for setting up the master mix solutions, i.e., preparations containing all reaction components except the nucleic acids to be tested. The objective is to prepare aliquots of the master mix solutions into the appropriate reaction format for a given assay and to store them until required for use. The suitability and stability of reaction components, especially polymerase enzymes, are to be checked (e.g.) by pilot reactions, and the results of the evaluations have to be documented. For "hot-start" techniques (enzyme addition after a first denaturation step at high temperature), the polymerases are also omitted from the master mix.

1.1.3. Clothing.

Protective clothing should preferably feature close-fitting sleevebands and should close tightly at the front. Surgical gowns are ideally suited for this purpose and are usually available, at least in a hospital laboratory. Throughout the entire work, workers must wear gloves and should change them frequently. When leaving the area, workers must leave their protective clothing behind.

An additional safeguard against contamination of specimens is the use of disposable operating caps. These are worn only rarely in routine practice, but their use should be strongly considered, particularly in this work area, where a contamination may ruin whole sets of stock reagents and master mixes.

1.1.4. Equipment.

Pipetting by mouth is strongly prohibited. Pipettes and pipetting aids must be autoclavable. Electric microdispensers for aliquoting increase the pipetting precision and thus limit any aerosol formation caused by frequent up-and-down pipetting.

The working area is to be equipped with a -20 °C freezer, a 4 °C refrigerator, a precision balance, a pH meter, a chipped ice maker, and stationery. The surfaces of the workbenches should be able to withstand decontamination procedures with such chemicals as sodium hypochlorite (3). Ultraviolet irradiation of work surfaces is also effective. Given the critical importance of the distance and the energy of irradiation for decontamination, we use in our laboratories pull-down UV lights (254 nm wavelength) that can be adjusted to within ~60–90 cm (~2–3 ft.) above the workbenches after the work in the work area is finished. Also, because of the small size of a few hundred basepairs and the fact that dried aerosols are less susceptible than "wet" aerosols to UV damage, PCR fragments must be irradiated for extended periods—optimally, overnight (4)(5)(6). Finally, a logbook (or a similarly suitable means of documentation) for recording use of the laboratory space and equipment has to be provided.

1.1.5. Consumables.

Consumables include cleansing liquid and disposable tissues, disposable gloves, disposable caps (where used), autoclavable reagent vessels of various sizes, disposable weighing paper, reagents for nucleic acid preparation, and reagents for reactions (e.g., enzymes, buffers, dNTPs).

1.2. sample/template preparation and cdna synthesis

1.2.1. Operations.

The sample preparation work area serves as specimen storage area. In addition, the extraction of nucleic acids (RNA, DNA), their storage, and their delivery into prepared reaction vessels (see above) are performed in this area. Single-strand cDNA synthesis for RNA analyses is also done in this work area.

1.2.2. Job description, flow of work.

The reagents and consumables from this working area must not be taken into the PCR pipetting area, because they might be contaminated with unamplified nucleic acids. Also, leftover master mixes, enzymes, or reagents must not be transported back to the reagent storage and set-up area. Sample preparation must not be started after previous work in the PCR product analysis area (see below).

Good pipetting technique is instrumental for untroubled amplification procedures. Because contaminations from aerosol formation may occur, unnecessary moving around the laboratory should be avoided. Aerosol contaminations from the access area to the sample preparation area can be reduced by positive pressure conditions inside the laboratory. To avoid cross-contamination between samples, reaction vessels containing reaction mixes must be closed after addition of the test nucleic acids. For potentially infectious materials, established recommendations for handling and disinfection must be observed.

Used pipette tips must be disposed of exclusively in suitable decontamination containers, e.g., containing sodium hypochlorite solution. Laboratory bench tops must always be cleaned at the end of work, and any spillage of test material must be recorded.

Appropriate UV radiation of laboratory tables (254 nm wavelength, short distance to work bench surface) is suitable for decontamination. For safety reasons, we recommend a UV radiation source that can be switched on/off either from outside the room or by a timer. A pull-down UV tubular lamp mounted over the laboratory bench can be used to assure efficient irradiation of laboratory bench surfaces after work.

Use of suitable commercial systems based on liquid extraction/precipitation, adsorption of DNA to silica surfaces, or anion-exchange chromatography permits fast and trouble-free recovery of sufficient quantities of DNA or RNA from a variety of specimen types. Alternatively, the nucleic acids can be prepared according to accepted procedures with home-made reagents (e.g., (7)(8)(9)). The recovery of RNA is performed in a manner similar to DNA preparation. However, important differences are implied by the instability of the analyte and the omnipresence and stability of RNases. Moreover, several DNA extraction methods and commercial preparation kits require a RNase digestion of the DNA sample. If these reagents are used in DNA extraction and both types of nucleic acids are handled in the same area, particular care must be taken to guarantee RNase-free conditions and solutions for RNA work by using dedicated consumables and pipettes.

For various reasons, it is practical to carry out a cDNA synthesis immediately after the RNA preparation. The cDNA synthesis should be carried out in the sample preparation area to help avoid contamination. Being more stable than RNA, the storage of a first-strand cDNA is less critical. Also, performance of the first-strand synthesis in the "downstream" working area for PCR assembly entails storage of the samples, because their transport back into the sample preparation area is, by definition, prohibited. To achieve the flexibility required for this RT reaction, one or more thermoblocks should be set up in the sample preparation area.

The optimal temperatures for cDNA synthesis depend on the enzyme chosen. One-step methods are preferable; i.e., use of heat-stable polymerases with RT activity under PCR buffer conditions (example 1, below) is safer than methods that follow cDNA synthesis by requiring opening of the reaction vessels for purposes of buffer adjustment (example 2, below) or polymerase addition (example 3, below). When using enzymes that possess both RT and DNA polymerase activity, one should assess the RNA dependence of the assay separately in the examination of intron-free genes as well as possible interference from processed pseudogenes. Examples of assay strategies currently in use are:

1) Addition of a polymerase with RT activity (e.g., Retrotherm from Epicentre Technology) → cDNA synthesis → no opening of vessels → PCR

2) Addition of a polymerase with RT activity (e.g., rTth from Perkin-Elmer) → cDNA synthesis → opening of vessels → adjustment of buffer to PCR conditions (here, chelate buffer) → PCR

3) Addition of RT (e.g., AMV-RT from Pharmacia) → cDNA synthesis → opening of vessels → addition of polymerase and PCR components → PCR

The cDNA copies of the test material are kept in the sample preparation area. PCR amplification from specimens is not allowed in this area.

1.2.3. Clothing.
Marked laboratory clothing as described above; frequent change of gloves.

1.2.4. Equipment.
Workbench with hood and UV radiator (see *Sections 1.1.4* and *3.2.2*) and positive-displacement pipettors or regular pipettors in conjunction with aerosol-proof disposable pipette tips. The pipettors should be autoclavable. Dispensers, freezer (-20 °C and -80 °C), refrigerator (4 °C). Additional equipment includes a vortex-type mixer and a waterbath or heating block. A logbook (or similarly suited means of documentation) must be available.

For RNA work, two additional pieces of equipment are recommended:

1) A cooling microcentrifuge (e.g., from Eppendorf) or centrifugation in a cold cabinet is preferred for RNA work for the following reasons: First, RNA preparations are best done on ice because of the low stability of the analyte and, depending on the specimen material, because the precipitation of RNAs is a usual step in preparation protocols (8). Second, when stored for extended periods of time, RNA is usually kept as an ethanol precipitate, also for reasons of stability, and requires centrifugation before use in RT reactions. Centrifugation in the cold minimizes the risk of degradation while handling RNA.

2) Depending on the method used and the specimen investigated, shearing of the high-molecular-mass DNA, commonly done by passing the samples through a syringe needle (8), may be necessary to reduce the viscosity of the material. However, this bears a substantial risk of contaminating the work area with unamplified nucleic acids, is hazardous with respect to handling, and is also impractical for routine purposes. Instead, high-molecular-mass DNA may be degraded by using a suitable ultrasonic water bath, obviating the need to open the sample tubes.

1.2.5. Consumables.
Cleansing liquids and disposable tissues, aerosol-tight pipette tips, autoclavable reagent vessels, gloves, disposable caps, reagents for preparation of samples (prepared in reagent set-up area).

1.3. pcr reaction mix assembly
1.3.1. Operations.
Dispensing of sample material (from sample preparation area, see *Section 1.2*) and master mix solutions (from reagent storage and set-up area, see *Section 1.1*) into the reaction mixtures as well as the amplification reactions is performed exclusively in this working area. In nested PCR assays, it is usually necessary to open the vessels after the first round of PCR. Consequently, nested PCR possesses a substantially higher contamination risk and therefore demands particularly strict attention. The setting aside and installation of this working area should be obligatory for all nested PCR applications.

1.3.2. Job description, flow of work.

Amplification of DNA fragments or of first-strand cDNA fragments is to be done exclusively in this working area. There should be no access from this section to any "upstream" working areas. Working under conditions of reduced atmospheric pressure is advisable to impede any leaking of aerosol contaminants from this area.

To avoid contaminations by aerosol formation, work should be set up beforehand, and any moving around the laboratory should be kept to a minimum. Pipetting should be performed in a hood. The opening of predispensed reaction mixes must be performed with great care, particularly between nested PCR steps. A good procedure is to briefly centrifuge all liquids in the reaction tubes before opening. Very small centrifuges (e.g., Picofuge$^{(TM)}$ from Stratagene) are ideally suited for this purpose; they take up very little bench space, are easily operated with one hand, and fit into most work cabinets. Moisture barriers, such as paraffin wax or light mineral oil, provide additional protection against contamination. However, one must be aware that mineral oil itself can be the cause of persistent contaminations. Used pipettes must be disposed of, preferably in containers filled with decontamination liquids (see *Section 3.2.2*). We also recommend analyzing samples in duplicates or amplifying two different DNA sequences.

Commercially available wax pellets can be used for hot-start assays. Alternatively, a chemically denatured (and, in this state, inactive) DNA polymerase (TaqGOLD$^{(TM)}$; Perkin-Elmer) has recently been introduced. Upon prolonged exposure to high temperatures near 94 °C, the enzyme will reactivate, thereby enabling an elegant hot start of PCR. For nested PCR, increased attention must be given to possible splashes when opening vessels. A brief centrifugation before opening reaction vessels is especially important. For pipetting purposes, reaction vessels should be opened under a safety hood.

Laboratory bench surfaces must always be cleaned and decontaminated after finishing work and at the end of the workday. Again, any spilling of solution is to be recorded.

1.3.3. Clothing.

Marked laboratory clothing as described above, and wearing of gloves and caps. In this area, gloves should be changed frequently. Clothes must be left behind when leaving the room.

1.3.4. Equipment.

Workbench with hood and UV radiator (see *Sections 1.1.4* and *3.2.2*), autoclavable pipettors, thermocycler, waterbath, stationery, electric micropipettor, and one or two Picofuges (see above).

1.3.5. Consumables.

Cleansing liquids and disposable tissues, aerosol-tight pipette tips, autoclavable reagent vessels, gloves, caps, and reagents for assay preparation (produced in pre-PCR area).

1.4. pcr product analysis

1.4.1. Operations.

Operations depend on the sensitivity (detection limit) of the method for detection of PCR fragments.

1.4.2. Job description, flow of work.

For detection of amplified PCR fragments, a variety of methods are available, including those listed here. The method to use depends on the analytical problem to be solved.

Agarose gel electrophoresis

Polyacrylamide gel electrophoresis

Nondenaturing

Denaturing gradient

Hybridization method (radioactive, nonradioactive)

Solid phase

Dot–blot

Southern transfer

In solution

ELISA

Sequencing methods (radioactive, nonradioactive)

Other methods

The analysis of amplification products inevitably leads to a contamination of this area by PCR fragments. In contrast to the other PCR working areas, the following safety aspects have to be considered to protect the laboratory personnel in this area:

1) Common use is made of mutagenic and toxic substances, e.g., ethidium bromide, acrylamide, formaldehyde, or radioisotopes (the pertinent radiation protection recommendations are applicable, including wearing of personal dosimeters, where necessary).

2) When handling fragments previously amplified from genes with an oncogenic potential, appropriate protective measures should be considered. Laboratory staff should be informed accordingly.

1.4.3. Clothing.
Normal laboratory and protective clothing.

1.4.4. Equipment.
Depends on method used.

1.4.5. Consumables.

Depend on method used.

2. Preanalytical Aspects

The techniques used in the amplification of genetic information require special emphasis and a clear definition of the preanalytical steps, especially when these techniques are applied to diagnosis. On the one hand, nucleic acids (especially RNA) can easily be destroyed through ubiquitous nucleases; on the other, the extremely high sensitivity of PCR necessitates the adoption of special precautions to protect the sample against contamination by unrelated nucleic acids (e.g., from laboratory personnel).

The quality of the test results is influenced to a high degree by factors that affect sampling and dispatch of samples. Aspects of sampling, sample storage before dispatch, and dispatch itself must be seen as important elements of the preanalytical stage. Minor mistakes in connection with preanalytical steps that are not standardized may have severe consequences for the test result. In the individual case it will be difficult, if not impossible, for the laboratory to reconstruct any such mistakes made by the sender.

In the following sections, sample preparation is differentiated from the preanalytical step and is defined as part of the analytical stage, because the choice of method depends on the intended application, and the sensitivity of the assay will depend on the preparation procedure.

2.1. specimens

In principle, PCR analysis can be applied to a wide variety of materials, including whole blood or bone marrow containing an anticoagulant such as EDTA or citrate, serum or plasma, dried blood (filter-paper cards), buffy coat, sputum, mouthwash, bronchial lavage, cerebrospinal fluid, urine, stool, biopsy material, cell cultures, fixed tissue, embedded tissue, tissue sections, and so forth. Because heparin inhibits PCR reactions, specific precautions have to be considered when the use of heparinized material is intended (see *Section 2.2*). Depending on the test material, pretreatment of the sample may be necessary before stabilization, e.g., liquefaction of sputum with hyaluronidase.

2.2. sampling

Sampling is best done in closed, disposable sampling systems, as is customary with other clinical test material. New disposable plasticware can be considered nuclease-free and used without further pretreatment. Where nonclosed sampling systems are used, e.g., for urine, secretions, stool, or bone marrow, or when particulate specimens like hair are obtained, special attention must be given to protecting the sample against contamination with (e.g.) hair, epidermal scale, or sputum from the sampler. At the very least, disposable gloves must be worn.

With respect to reusable general glassware, the following should be considered: Glassware should be autoclaved or even better, heat-sterilized, because *(a)* sterile glass equipment is not necessarily free of

contaminating DNA, and *(b)* glassware is often a source of highly resistant RNA-degrading enzymes, a major source of which is the hands of the investigator. RNases can be permanently inactivated through high temperatures, e.g., by baking at 250 °C for 4 h or more (9).

In blood and bone marrow specimens, clotting must be inhibited. EDTA and citrate are commonly used and are the preferred inhibitors. The citrate-containing specimen collection systems (also used for routine blood coagulation tests) will dilute the specimen by 10%. In contrast, heparin (routinely 14.3 IU/mL of whole blood) reportedly inhibits amplification in concentrations as low as 0.05 IU per reaction volume (10). For heparinized specimens the following must be taken into consideration:

1) For simple PCR tests not requiring high sensitivity, dilution of the prepared nucleic acids is ordinarily sufficient to overcome the inhibition. If heparinized material has to be used and a more sensitive DNA PCR is required, nucleated cells should be isolated first and washed repeatedly in physiological buffers before further processing.

2) Where highly sensitive RT-PCR methods are required, additional measures are necessary to overcome heparin inhibition. Methods shown to fail at this are boiling, Sephadex chromatography, pH shifts with subsequent gel filtration, repeated ethanol precipitations, and treatment with protamine sulfate. Although treatment with heparinase restores the amplification (11)(12), this enzymatic purification step is costly. In addition, RNA may be degraded during enzyme incubation by traces of RNase still present in the sample or by heparinase preparations contaminated with RNase.

3) As demonstrated recently, lithium chloride can separate heparin from RNA, thus reversing the inhibition. This method, which reliably restores amplification from heparinized blood samples, is easily incorporated into a routine RNA preparation procedure without additional effort (13).

2.3. sample fractionation

When necessary, target cells can be enriched before sample stabilization and dispatch. Enrichment is useful when specimens contain low numbers of cells (e.g., urine, ascites, secretions, excretions). The sample should be centrifuged at low speed before stabilization.

In specimens containing high numbers of nonnucleated cells, e.g., blood or red bone marrow, stabilization may be preceded by a selective lysis of erythrocytes and followed by recovery of the nucleated cells through centrifugation at low speed. Another common method of sample fractionating is Ficoll density-gradient centrifugation. When performed at the site of specimen sampling, problems may arise from poor standardization, variable recovery of target cells, and danger of specimen contamination. Problems with standardization of Ficoll gradient enrichment of nucleated cell populations may be circumvented in the future by using combined sampling/fractionating systems, e.g., the Vacutainer Tube CPT tubes(TM) (Becton Dickinson).

2.4. sample stabilization

Stabilization of test material is essential because nucleic acids degrade rapidly and is especially important when RNA has to be analyzed. Instant inactivation of DNases and RNases is reliably achieved

by chaotropic substances (especially guanidinium isothiocyanate, GITC). GITC has been increasingly used in concentrations of 4 mol/L as originally described (8). Organic solvents, e.g., phenol, may be added in parallel. Extraction systems based on these additives are now commercially available, e.g., RNAzol, Trizol. However, the limited stability of reducing agents (ß-mercaptoethanol or dithiothreitol) and their requirement for sample stability need to be considered. Therefore, the user must be aware that batches of ready-to-use extraction solutions have a limited shelf life because of instability of some of their individual components, e.g., ß-mercaptoethanol. Moreover, the handling of organic solvents is hazardous to human health and due care must be taken during handling.

At the site of specimen collection, original or enriched material is lysed by addition to reagent tubes containing GITC. The appropriate concentration leading to an irreversible denaturation of RNases has recently been determined to be 5 mol/L. Use of GITC concentrations <4 mol/L leads to very rapid RNA degradation (14). After proper stabilization, the material usually need not be cooled before mailing for analysis. At temperatures cooler than room temperature, GITC will crystallize, thereafter requiring complete thawing before addition of the specimen. Depending on the abundance of the RNA target to be amplified, chelating agents, e.g., citrate (combined with low temperatures), may be appropriate to inhibit RNases, given that the enzyme activity of the RNases depends on free divalent cations (9). In our experience, GITC is preferable where maximum sensitivity is required and where delays in transport to the laboratory cannot be excluded. In any case, the suitability of a stabilization system should be documented with respect to the sensitivity of the subsequent RT/amplification reactions.

For extraction of DNA from leukocytes, blood containing EDTA as anticoagulant requires no special stabilization; nonetheless, samples should be dispatched without delay to the laboratory.

2.5. sample dispatch

Samples stabilized appropriately may be dispatched by regular mail at ambient temperatures. This applies to EDTA-containing whole blood for DNA preparation and GITC-stabilized specimens for RNA recovery. Cooling is not necessary but depending on the application, prolonged storage at room temperature will result in a critical loss in sensitivity (14). In general, samples should be dispatched in breakproof containers. RNA targets to be investigated in nonstabilized samples must be shockfrozen and then dispatched in solid CO_2. Samples that reach the laboratory in the state of thawing should be invariably rejected.

2.6. sample storage

Specimens for DNA analysis should be stored in buffers of 10 mmol/L Tris, 1 mmol/L EDTA (pH 7.5–8.0), at 4 °C. Specimens for RNA analysis should be kept in buffered solution preferably at -80 °C or in liquid nitrogen. Equally suitable is storage as an ethanol precipitate at -20 °C. GITC-stabilized RNA samples may be stored for ~7 days at room temperature. In cases of longer storage, less-sensitive limits of RNA detection have been observed. Such findings must be taken into account when only a few viruses or cells are to be detected.

3. Factors Interfering with Analytical Procedures

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3.1. preparation of specimens

RNA or DNA (e.g., from human cells or viruses) may be isolated from a multitude of different specimens (see *Section 2.1*). For DNA analysis, no special measures are usually necessary if sample transport to the laboratory and DNA preparation are performed without delay. When RNA has to be analyzed, degradation of the analyte may be prevented through stabilization as described above.

3.1.1. Interferences related to DNA preparation.

DNA preparations of inferior quality are often characterized by incomplete removal of inhibitors, either from the sample itself (e.g., heme, its precursors, or degradation products (7)) or introduced during inadequate sampling (e.g., heparin *[10]*). When phenol is not completely removed, the subsequent enzymatic steps (e.g., PCR, restriction digest) may be inhibited (15). In this case, the DNA should be taken up in a larger volume of buffer before phenol extraction, chloroform extraction, and repeated precipitation. Although usually not required, traces of chloroform may be removed by ether extraction (9). When starting with very small amounts of sample material (e.g., Guthrie filter cards, tissue sections), losses in nucleic acid may occur during preparation. Such losses can be minimized by adding carrier tRNA (Gibco BRL) or glycogen (Boehringer Mannheim) during the precipitation steps (9). Other reagents of suitable quality (molecular biology-grade) can be purchased from several commercial sources. Where used according to manufacturer's instructions, such reagents do not interfere with the subsequent enzymatic reactions.

Long-term storage of DNA should be done exclusively in buffered solutions. Tris–EDTA buffer (10 and 1 mmol/L, respectively, pH 8.0) is well suited. In water, autocatalytic processes through depurination can result in a completely degraded DNA after only a few weeks. DNA appropriately prepared may be stored in buffered solutions at 4 °C for years without any large losses in quality or quantity.

3.1.2. Interferences related to RNA preparation.

The sensitive detection of intact RNA is frequently compromised by inhibitors that have not been eliminated completely, e.g., heme and heparin, as described above. RNA degradation presents a major problem. Common causes for a failure of RT-PCR assays are insufficient sample stabilization before sample dispatch and RNase contamination of reagents for preparation. For the former, the logistics of the preanalytical phase need to be checked; if there is evidence for RNA degradation, the specimen should be rejected by the laboratory, which should order a new specimen and give detailed instructions to the sender regarding the proper procedure. For the latter, use of commercial preparation kits, quality-checked by manufacturers, is recommended for routine tests.

In addition, RNA may be lost during precipitation or long-term storage of prepared RNA. A loss, especially of polyA-mRNA, may be prevented through coprecipitation with glycogen (10 g/L in diethylpyrocarbonate-treated water), which gives a clearly recognizable visible precipitate. Because RNA is unstable in water, long-term storage as an ethanol precipitate at -20 °C or -80 °C is recommended.

3.2. reverse transcription and amplification of target sequences

3.2.1. Interferences with cDNA synthesis.

cDNA synthesis is the first enzymatic step in RT-PCR. The cDNA generated as the reverse complement from the target mRNA then serves as the template for the subsequent amplifications.

cDNA synthesis is convenient when the amplification of single exons from DNA is not feasible, when no information is available regarding genomic sequences or gene organization, or when disease-associated or disease-specific transcripts are to be detected. In addition, analysis of a few cDNA fragments instead of many genomic fragments may be advantageous. For prospective studies, it may be safer to translate mRNA into cDNA to achieve better conditions for long-term storage.

The following factors commonly affect the efficiency of cDNA synthesis:

1) Reduction or complete absence of RT activity. Inadequate cDNA synthesis caused by low enzyme quality, decomposed reagents, or pipetting errors must be excluded.

2) Inhibitors of the RT or of the heat-stable polymerase (e.g., phenol, heme). These are to be suspected when no amplification can be achieved even though the RNA appears to be intact. Amplification of a sufficiently expressed housekeeping gene (e.g., β_2-microglobulin, glyceraldehyde-3-phosphate dehydrogenase) may serve as control if the sample, and thus the inhibitor, is appropriately prediluted.

3) Degradation of the RNA (which can be checked through appropriate controls during or after preparation). A degraded mRNA may be assumed when the ribosomal RNA populations 28S, 18S, and 5S are no longer clearly defined, or are possibly completely absent. A quick check with electrophoresis in a nondenaturing, ethidium bromide-prestained agarose gel is usually sufficient. This simple test requires ~1 µg of total RNA for reasons of detectability; thus it is not suited for all methods. A more thorough assessment of whether the RNA species is intact requires agarose gel electrophoresis under denaturing conditions.

4) Contamination of RNA with genomic DNA. Primers for the amplification of cDNA fragments usually can be positioned to hybridize in different exons, which will warrant RNA-dependent amplification. Where this is the case, PCR products from contaminating non-mRNA nucleic acids, should they occur, are larger than those obtained from cDNA. Where this design is not possible, e.g., with intron-free genes or intron-free pseudogenes, the RNA preparation is treated with RNase-free DNase. For such targets, an amplification without a preceding RT step must be performed to control sufficiency of this step. This control is not recommended when one is using enzymes that possess both RT and DNA polymerase activity (e.g., rTth).

5) Residual DNase activity. If RNA samples are treated with RNase-free DNase before first-strand cDNA synthesis, care must be taken to remove the DNase activity before the reverse transcription step. Usually, 10 min at 65 °C is sufficient and will also aid in breaking up secondary structures in the RNA template that can interfere with efficient reverse transcription (9). The RNA sample can be extracted with organic solvents/precipitation, but this may result in loss of material or inhibition of the subsequent

enzyme step because of trace amounts of phenol in the reverse transcription reaction.

6) Insufficient priming of mRNA in the first-strand cDNA synthesis attributable to, e.g., the type of priming. Three different methods for the initiation of first-strand cDNA synthesis exist: oligo(dT) priming, random priming, and transcript-specific priming. *Oligo(dT) priming* starts the reverse complementary first-strand cDNA synthesis at the 3'-end of the mRNA. An unfavorable secondary structure of the mRNA or a long mRNA sequence can lead to the first-strand cDNA being not fully reverse-transcribed towards the 5' end of the mRNA. Consequently, amplification is not successful in the subsequent PCR amplification, even if the mRNA has been successfully prepared from the specimen. *Random priming* starts cDNA synthesis from short primers with random sequences (hexamers or octamers). The cDNA synthesis initiates at random sites and, theoretically, will thus cover all RNA species present in the sample. The average length of the cDNA depends on the molar ratio of random primers and mRNA, with a high excess of random primers favoring short DNA fragments. If this is the case, subsequent PCR amplification may be compromised. For *target-specific mRNA priming*, the suitability of the respective 3' primer for cDNA synthesis has to be established.

3.2.2. Factors affecting PCR.

Various factors can lead to false-negative or false-positive results in PCR, e.g., inhibitors or the absence of enzyme activity (see above), inappropriate annealing temperature, suboptimal magnesium concentration, or contamination of patients' samples or reagents, each of which will be discussed separately.

Of particular importance is the selection of the proper primer pairs. Usually, commercially available primers are high in quality, especially when further purified by the vendor, e.g., by HPLC chromatography. Today, numerous 5'-end modifications can be ordered, allowing labeling of the oligonucleotides with fluorochromes, biotin, amino linkers, and others. If one wants to design a test "from scratch," however, much thought should be given to the initial primer design. Several computer programs—either part of a regular DNA analysis software package or a stand-alone program that can be ordered—can help avoid unfavorable secondary structures, predict the occurrence of primer dimer formation, allow approximations of optimal annealing conditions, and so forth. (Evaluation of the algorithms underlying primer calculation or program features is beyond the scope of this document.) Once a primer is identified, the investigator should "run" it through the genetic database to check for cross-hybridization. This can be done conveniently through the Internet by using the BLAST programs displayed in the National Center for Biotechnology Information homepage (http://www.NCBI.NIH.NLM.gov). Certainly, however, the performance of a chosen primer pair identified through a software still requires careful evaluation with respect to specificity and amplification efficiency during the phase of test establishment.

Inhibitors of PCR are to be avoided in the preanalytical phase and have to be eliminated during sample processing within the laboratory.

The annealing temperature is dependent on the sequence, i.e., length and base composition, of the amplification primers. In assessing the optimal temperature, one may apply the Wallace rule as a rule of

thumb; i.e., the melting temperature of an A–T bond is in the range of 2 °C and that of a G–C bond is in the range of 4 °C. As with the use of computer programs (see above), the optimal primer performance always has to be investigated systematically by suitable pilot experiments.

The optimal magnesium concentration has to be established by appropriate titration experiments. The optimal concentration window may be very narrow. As a rule of thumb, one should initially start by using the standard concentration recommended by the manufacturer of the enzyme. Because the free magnesium ion concentration determines the efficacy of the enzymatic nucleic acid polymerization, the nucleic acid content or the individual concentrations of other magnesium-binding polyanions in the preparation will influence the amplification result. An optimal concentration of magnesium should result in a maximum yield of PCR, and no unspecific bands should be detectable by agarose gel electrophoresis.

Uniform temperature transition is an important aspect for a successful amplification. For example, the wall thickness, the material making up the reaction vessels, and a good fit of the reaction vessels into the thermoblock well are essential.

The homogeneity of heat conduction in the reaction block is of crucial importance. The heat performance of the cycler and the uniformity of heat conduction in the heating block must be controlled regularly to avoid false-negative results.

During test evaluation or in case of uncertain interpretation, the identity of the PCR product must be certified by proper means, i.e., by restriction fragment polymorphism, hybridization, or, ideally, by DNA sequencing of the purified PCR fragment.

3.3. contaminations

For practical reasons, the following types of contamination are distinguished here: contamination with PCR fragments (product contamination); contamination with native, genomic DNA; reagent contamination (stock solutions or working solutions); and cross-contamination (e.g., spreading of aerosols from a positive sample into an originally negative sample).

For all amplification techniques, greatest attention is directed towards the prevention of contamination, because locating its source around the laboratory is time-consuming and tedious. Once a contamination has occurred, testing has to cease, until the source is identified. Without exception, test results must be rejected, even if only one of the accompanying contamination controls reveals contaminating PCR fragments.

3.3.1. Contamination sources in the preanalytical phase.

Nucleic acids not originating from the patient may contaminate the test material during the preanalytical phase.

3.3.2. Contamination sources in the analytical phase.

As a general rule, contamination of the sample may occur anywhere during the different steps of the analytical phase. Accordingly, any component of the reaction mixture and any piece of laboratory

equipment coming into contact with the reaction during nucleic acid preparation and reaction set-up is suspicious as a source. Examples include: contaminated reagents (e.g., bovine serum albumin, gelatin, or mineral oil); commercially available enzyme preparations (16); consumables (e.g., reaction vessels, pipet tips); and laboratory equipment (e.g., pipettors, centrifuges).

One origin of contamination is cross-contamination with unamplified DNA during the simultaneous preparation of many specimens. Most contaminations, however, will consist of the specific PCR fragments generated during previous amplifications.

A contamination of reagents, consumables, or laboratory equipment used in the first three work areas (see *Section 1*) may indicate improper laboratory procedures. In contrast, contamination of the product analysis area is inevitable when PCR products are pipetted. Great care must be taken to identify this sort of contamination. Specifically, one main source is the microdrops that cross-contaminate samples, e.g., when these are loaded onto agarose gels or onto dot–blot equipment. Whether or not such contaminations are detected and thus influence the test result depends on the detection limit of the method used to detect the PCR fragments. For example, a contamination not detected in ethidium bromide-stained agarose gels may very well become apparent if the method is changed to a sensitive hybridization protocol. Multiple analyses of the same sample, run at different positions on a gel or in different runs, will help to exclude such cross-contaminations as the cause.

3.3.3. Avoidance of contamination.

As a general rule, prevention rather than removal must be given highest priority. Individual aspects have already been dealt with earlier in the context of dividing the working areas and the logistical separation of assay steps.

Several methods exist to destroy amplification products generated in previous assay runs before they can evolve into a detectable contamination. As a general safeguard, for example, amplification reactions can be performed with reaction mixes, in which dTTP is partially replaced by dUTP, thereby generating uracil-containing specific amplification fragments. A preamplification digestion of reaction mixes with uracil-*N*-glycosylase will therefore destroy contaminations carried over from previous assays (17). An alternative "post-PCR sterilization" is photochemical generation of DNA adducts by isopsoralen compounds in the presence of longwave UV light (18)(19). These also prevent contamination, because the DNA adducts are refractory to amplification but do not interfere with post-PCR hybridization procedures.

However, the aforementioned methods are not generally recommended and should be viewed with caution, because they can generate a false feeling of safety. In particular, these measures will not prevent contaminations from foreign, nonamplified native DNA.

3.3.4. Decontamination measures.

Efficient decontamination at regular intervals after termination of work is mandatory. A combination of various methods promises the best results. Decontamination measures include, but are not limited to: chemical cleaning of surfaces with 100 mL/L sodium hypochlorite (3); permanent UV radiation (254

nm) of laboratory benches and other surfaces after use (4); autoclaving of laboratory equipment, e.g., pipettors; and flaming of laboratory equipment, where possible.

3.4. analysis of amplification products

Various techniques can be used to evaluate amplification products, including electrophoresis, restriction digestion, blotting, hybridization, sequencing, and mass spectrometry, each of which may be subject to specific disturbances. Assessing the specificity of a PCR only on the basis of the product's fragment length determined by simple agarose gel electrophoresis is to be reserved for well-established assays.

3.4.1. Interferences in electrophoresis.

In general, two different types of electrophoretic separation techniques are used in molecular biological diagnosis: submarine agarose gel electrophoresis and polyacrylamide gel electrophoresis. Both techniques can be applied under either denaturing or nondenaturing conditions. As with the electrophoretic characterization of proteins, the following factors affect the electrophoretic characterization of PCR fragments:

1) False gel concentrations, ionic strength, or pH. A careful control of reagents or the use of commercially available ready-to-use reagents checked for their quality by the manufacturer is suggested.

2) Imperfect sample preparation. High salt content of the sample (e.g.) can affect the electrophoretic mobility and the band pattern of the DNA fragments and may lead to incorrect estimation of the fragment size.

3) Overstaining DNA products or using insufficiently sensitive stain. The intercalating fluorescent stain ethidium bromide can differ in sensitivity between manufacturers or lot-to-lot. The right stain concentration is easily established through use of amplification products as controls. (*Note:* Intercalating DNA stains are strong carcinogens; therefore, contaminated buffers must be properly disposed of.)

3.4.2. Interferences related to restriction digestion.

Restriction digestion may precede a PCR reaction so as to increase the specificity of the reaction (e.g., cutting a processed pseudogene in a RT-PCR) or to detect the methylation of target sequences (e.g., X-inactivation). The main application of restriction digestion, however, is either to verify the specificity of a PCR product or to detect gene mutations via defined restriction fragment lengths (restriction fragment length polymorphism, RFLP). In comparison with restriction of unamplified DNA, the following must be considered when amplification products are to be digested:

Usually, the unit definition, as given by the manufacturer, is the basis for assessing the enzyme concentration required for the restriction digestion. One unit is defined as the quantity of enzyme that completely cuts 1 µg of DNA at a defined temperature within 1 h. The abundance of a restriction site in the test DNA—assuming a uniform base distribution—statistically depends on the length of the recognition sequence of the enzyme: e.g., for a recognition sequence of 4 bases, 1 site/4 = 256 bases; for a recognition sequence of 6 bases, 1 site/4 = 4096 bases. Obviously, this does not apply to a PCR product, because the "cutting site density" in the digestion reaction is very high compared with the naturally occurring sequences, thereby rendering the mass-related enzyme concentration a limiting

factor. Accordingly, the unit definition is not valid for amplification products, and the amount of enzyme necessary for digestion must be determined empirically. The manufacturers provide information on the characteristics of restriction enzymes, e.g., inhibition by glycerol or star activity, and the related quantity per volume to be used.

Further major complications are insufficient or absent activity of the restriction endonuclease, possibly caused by the presence of certain compounds in the amplification reaction mix. For example, the salt conditions required for cutting may be incompatible with the salt conditions used for amplification. If in doubt, use of organic solvent extraction and subsequent precipitation most often will solve a cutting problem.

Another cause for insufficient or absent digestion is decreased activity of these temperature-sensitive enzymes.

The assessment of a quantitative restriction digestion must be assured through appropriate internal and external controls, because a partial digestion can entail misinterpretations of the banding pattern.

3.4.3. Interferences related to nucleic acid transfer (blotting).

There are essentially two reasons for the transfer and immobilization of DNA from a gel to a solid matrix (e.g., nylon membrane, nitrocellulose), namely, to increase the specificity of detection of a PCR product and to decrease the detection limit relative to simple staining with fluorescent dyes.

Even in cases of a professional performance of blotting, the presence of membrane regions in which the binding of nucleic acids is reduced or even absent may compromise results. Avoidance of membrane artifacts is a demand addressed to manufacturers.

3.4.4. Interferences related to hybridization.

Inadequate hybridization results can be caused by inappropriate gene probes, inappropriate labeling methods, inadequate labeling of probes, or inappropriate hybridization or washing methods.

The gene probes most commonly used are: DNA fragments, synthetic oligonucleotides, and in vitro transcripts (antisense RNA probes). Probe production or labeling is mostly done with commercially available enzymes that are suitable for the respective labeling strategy. The enzymes most frequently used are: Klenow polymerase; T7 DNA polymerase; thermostable enzymes, e.g., Taq polymerase; T_4 polynucleotide kinase; terminal deoxy nucleotidyltransferase (tdT); T7 RNA polymerase; and SP6 RNA polymerase.

The efficiency of the labeling of a gene probe must be determined after each labeling reaction, because the sensitivity of the hybridization and thus the possibility of false-negative findings depends, among other factors, on the specific activity of the hybridization solution.

With radioactive labeling, the activity of a fraction of the labeled preparation is measured in a ß-scintillation counter. The specific activity of the gene probe (counts/minute per microgram of probe) is

then adjusted according to the total incorporated counts and the amount of probe used for labeling. For example, radioactively labeling a synthetic oligonucleotide by using T_4 polynucleotide kinase with [γP] ATP typically generates a specific activity of 1 x 10 to 5 x 10 counts/min per microgram. With nonradioactive labeling, the specific activity can be determined via a dilution series with subsequent detection in dot–blot. With both labeling methods, one must make sure that none of the measured signal is from non- incorporated label, e.g., radionuclide, fluorescence label, or a biotin-derivatized base.

To ensure reproducibility, the specific activity at time of labeling, the age of the probe at time of use, and the quantity of probe used in the hybridization preparation all must be recorded.

Hybridization conditions must always be ascertained empirically in the laboratory when a particular probe is first introduced into the test program. Accepted conditions as well as melting temperatures computed by oligonucleotide software programs can usually be used for a first approximation during the evaluation process. We recommend strict adherence to a fixed hybridization program once it has been established.

Both a temperature too low or an ionic strength too high will reduce the stringency of hybridization and may negatively affect the specificity of the detected signal. In contrast, raising the temperature, decreasing the ionic strength, or both, will increase stringency. Thus, a tight control of temperature and of reagents is the prerequisite to avoiding false-positive as well as false-negative results. Note that temperature and ionic strength should never be changed at the same time.

3.4.5. Interferences related to sequencing.

DNA sequencing, the most accurate method of determining the primary base composition of a DNA fragment, is imperative when controlling the authenticity of PCR products and has the highest analytical specificity for detection of point mutations in PCR products. Sequencing should be performed directly from the amplification reaction after PCR.

Because of absent 3' exon nuclease activity, Taq polymerase and some other heat-stable polymerases cannot correct primary incorporation errors (absent proof-reading activity). According the amplification conditions used, misincorporations of nucleotides into the growing DNA chain (Taq polymerase errors) will occur with various frequencies. A considerable fraction of the DNA molecules generated will differ from the original sequence as a result of these misincorporations. For Taq polymerase, the magnitude of these substitutions ranges from ~2 to 4 nucleotides per 10 000 synthesized bases. Other enzymes will have different error rates.

A misincorporation randomly introduces a mutation into the sequence that will subsequently be amplified during the remaining PCR cycles. Still, at the end of the amplification run, errors will be underrepresented in comparison with the authentic sequence at any position in the fragment, unless a very low target number was amplified in the first place and the misincorporation occurred within the first cycles. Because the risk for the latter will increase with the length of the DNA segment amplified, the amplification of shorter fragments should be favored. During direct sequencing of the fragments in the PCR reaction, the authentic signal will be the highest at any given position in the amplified fragment.

In contrast, when the amplification products are cloned into plasmids and the recombinant clones (harboring individual PCR amplicons) are subsequently sequenced, incorrect base sequences will result as a consequence of the misincorporations "singled out" by the cloning process. The importance of this problem is aggravated for assays that are designed for the amplification of a very low number of target nucleic acid molecules, the use of a high number of cycles, or both.

Enzymes with low error rates, so-called high-fidelity (or proofreading) polymerases, are commercially available. They should be used for amplification where the cloning of the PCR product is desirable and the probability of isolating the authentic gene segment has to be guaranteed. However, these enzymes are generally more expensive and do not possess the same processivity as the nonproofreading enzymes.

In summary, only the sequence analysis determined directly from the uncloned PCR products (direct sequencing) can determine the correct DNA sequence and is the method of choice for detecting mutations in a heterozygous state.

Poor template quality, defective sequencing reagents, or poor primer specificities are easily detectable by the ambiguous or absent sequence ladder. Where nonradioactive sequencing methods and software-supported base-calling is used, the software design or settings should be appropriate to allow the identification of samples with heterozygous genotypes, i.e., allelic polymorphisms or point mutations. Towards this end, sequencing reactions must be optimized for low background signals. Results that are not unequivocally clear must not be used for interpretation; instead, the procedure needs to be repeated. While it is a good idea for increased confidence to routinely sequence both strands of the amplified fragment, sequencing of the opposite strand is mandatory when problems with the base-calling persist.

4. Quality Controls

As mentioned above, a variety of controls should be implemented to assess the quality of single steps during analysis, e.g., for RNA integrity, its suitability for amplification, sensitivity. With increasing automation and standardization, the number of controls to be performed has to be reassessed. As in conventional clinical chemistry, internal and external quality control can be distinguished.

4.1. internal quality control

A considerable number of controls must be carried out in the laboratory to monitor the complex steps during DNA or RNA analysis. Such controls are mandatory for arriving at a conclusive interpretation of findings and serve to avoid false-positive and false-negative results. The production of reagents, the preparation of test material, PCR preparation itself, and each of these steps in its own right require thorough control because of the high analytical sensitivity of the PCR; the same applies, with appropriate alteration of details, to other enzymatic steps that may precede or follow amplification reactions.

4.1.1. Controls related to preparation of the test material.

For control of DNA preparation, agarose gel electrophoresis is most commonly used. The average length of the DNA is ~100 kb in routine manual preparation methods. In DNA preparation kits suitable for PCR, the average range is between 30 and 40 kb. However, clearly degraded DNA also generates strong fluorescent signals after electrophoretic separation and staining with ethidium bromide in a lower-molecular-mass range between 1 and 10 kb. Through digestion of the DNA with a methylation-insensitive restriction endonuclease, e.g., *Eco*RI, followed by electrophoretic separation, one can control for inhibitors of enzymatic activities (in the presence of inhibitors, high-molecular-mass fragments remain uncleaved). The presence of potential inhibitors is usually assessed by using photometry at 260 and 280 nm. In a good DNA preparation, the A_{260}/A_{280} ratio should be in the range of 1.75–2.0; otherwise, the contamination (e.g., with residual protein or phenol) may be too high. Photometric measurement alone does not allow conclusions to be made regarding the integrity of the DNA.

The fastest method for controlling the quality of the total RNA preparation is agarose gel electrophoresis under nondenaturing conditions, as was applied in the separation of DNA. In case of doubt, however, the RNA should be run on an agarose gel under denaturing conditions to check its integrity. Ideally, the three major ribosomal RNA species (28S, 18S, and 5S) will be detectable as relatively sharp bands. Bands smeared to lower molecular masses or absence of the bands strongly indicates decay of RNA. A densitometric measurement of ribosomal RNA bands with indexing may become an in-laboratory standard for assessing the quality of RNA preparations; assessment of peak asymmetry with peaks trailing to lower molecular masses also is a suitable indicator of RNA integrity. In addition, agarose gel electrophoresis will indicate the degree of DNA contamination in the RNA preparation. For these reasons, the photometric measurement alone does not allow one to draw conclusions as to the integrity of the RNA.

4.1.2. Controls for cDNA synthesis and amplification.

Preparations for PCR amplification must be controlled carefully with reaction-internal as well as reaction-external positive control specimens to check for enzymatic activity and to exclude the presence of putative inhibitors. Negative controls are essential for monitoring contaminations. In general, the following control reactions should be distinguished: The positive-control reaction supplies information about enzymatic activity and inhibitors in the sample; the negative-control reaction detects contamination with human DNA (e.g., from the investigator) or with PCR product; and the reagents-control reaction indicates whether any of the reagents are contaminated.

4.1.2.1. cDNA synthesis. The crucial control for monitoring the performance of cDNA synthesis is the use of an internal reaction control. cDNA synthesis can proceed either from mRNA transcripts, which are present in each RNA preparation (i.e., mRNA of ubiquitously expressed, so-called housekeeping genes such as transcripts for ribosomal proteins), or from RNA, which is added to the sample as an internal standard at the time of preparation. Internal controls for synthesis are positive controls that lead to a defined product; if the amplification is not successful, the controls indicate a degradation of the RNA, faulty priming during the cDNA synthesis, or absence of enzyme activity.

The addition of a defined number of molecules of an amplification control permits determination of the

lesser sensitivity of the RT-PCR. This is particularly important in PCR applications of high sensitivity. A mandatory contamination control for RT-PCR assays consists of a reaction with the RT omitted. In summary, equivalent amplification characteristics of control and template must be assured, and the control must be clearly distinguishable from the template.

4.1.2.2. PCR reaction. *Internal reaction controls* are positive controls and are particularly important in cases where the presence or absence of an amplification product is diagnostically relevant, i.e., with gene deletions or Y-chromosomal sequences. The negative result must be clearly distinguishable from a technical failure of the assay. Thus, to guarantee that amplification is successful under a given set of reagents and DNAs, one can use a control target gene essential for the organism to survive. Such genes are present in at least the hemizygous state, because their absence from the genome is lethal (i.e., so-called lethality factor). A good example is the gene for the vitamin D-binding plasma protein (20); its polymorphisms have been extensively examined in many ethnic groups worldwide, and no homozygous loss of gene activity has ever been identified. Therefore, a coamplification of a segment of this gene by the PCR will be in all cases successful and will demonstrate the successful amplification reaction.

External positive controls of appropriate DNA and appropriate dilutions thereof allow the quality of the reaction solution to be checked, and information regarding the detection limit and specificity of the PCR are obtained. The same master mix solutions used for the diagnostic test (i.e., patient's material) must be used in the external positive controls. Amplification controls are to be performed with each reaction if the detection limit of the procedure is suspected to be inadequate to detect the product. Controls of this type increase the contamination risk in the test series, if the nucleic acid used corresponds to a positive control sample. Vector-cloned target sequences or a genomic DNA of known copy number is suitable.

An *external negative control (contamination control)* must be performed in each PCR test; indeed, several controls should be contained in one run, one for each primer pair applied. In general, these controls are to furnish information about the point in time at which a contamination occurs in the course of a PCR-based test. A blank reaction vessel taken through the entire course of the sample preparation comes into contact with all solutions used in the preparation, but contains no amplifiable material (so-called mock preparation). If necessary, different mock preparations can be integrated at various preparation stages in the course of the nucleic acid preparation, if necessary. In this manner the individual steps at which contamination can occur can be identified. Mock controls allow assessment of the overall quality of the PCR test.

In addition, during the pipetting process, when bringing the samples into contact with the enzymatic amplification solution, water samples have to be added: These contain all reaction components, but water is used instead of the sample. Water samples must be present at least at the start and at the end of a pipetting series in which sample material is processed. The widely practiced method of detecting the absence of contamination solely by means of the water sample is not admissible, because this does not detect contamination sources in the course of sample handling; water can serve as a control only of the amplification reagents.

If the same amplification assay is to be repeated—as will be the case in most diagnostic tests—all

contamination controls must be checked regularly by means of specific hybridization with use of Southern transfer or dot–blot. In PCR tests that amplify RNA targets, a contamination control should be performed with the RT step omitted. In this way contamination caused by DNA fragments from previous amplifications can be detected.

4.1.3. Controls for the evaluation of test results.

4.1.3.1. Control of restriction digestion. Digestion of genomic DNA can be reduced by inhibitors of the activity of enzymes present in the preparation, by inappropriate reaction conditions, or by low enzymatic activity. The digestibility of genomic DNA can be assessed through agarose gel electrophoresis. The following criteria serve for an assessment of a successful restriction:

1) After restriction digestion with most enzymes, a continuous, mostly smeared band is observed from the high- to low-molecular-mass range. This reflects the size heterogeneity of the restricted genomic fragments. Where digestion is not successful, a pronounced high-molecular-mass fraction will persist at the molecular range corresponding to the uncut DNA. With some enzymes, a high-molecular-mass fraction may be observed even after extended digestion incubation times. Thus, although disappearance of the high-molecular-mass fraction indicates full enzymatic activity, persistence of this fraction does not necessarily indicate insufficient digestion.

2) An elegant method to assess inhibitors in the sample is to branch off an aliquot of the digestion mix, and then add to this a known amount of high-molecular-mass molecular marker (see *Section 4.1.3.2*). For example, together with the genomic nucleic acids, the 48-kb large λ-bacteriophage (e.g., 1 μg) is digested into its predicted fragments. One can monitor the suitability of the sample DNA on the basis of the digestibility of the indicator DNA in the aliquot by agarose electrophoresis after different time intervals and extend the digestion times of the genomic DNA if necessary (9)(21).

3) The human genome contains repetitive sequences, e.g., mitochondrial DNA, which may appear as distinct sharp bands (satellite bands) within the background smear of heterogeneous fragments. Satellites, therefore, do indicate successful restriction; however, not all enzymes will generate a satellite band pattern.

Restriction digestion of PCR products is often applied to identify mutations on the basis of various restriction fragment lengths. Compromised enzymatic activity in these cases leads to false interpretation of results. Complete digestion of PCR products can be controlled only by a second invariant restriction site of the same enzyme in the PCR product to be tested. This must be strictly observed in the construction of the diagnostic fragment, to assure a correct interpretation of results.

4.1.3.2. Control of electrophoresis. Quality assessment of electrophoretic methods involves, in the first place, calibrators for length and suitable control fragments at defined concentrations; controls must be subject to the same sample preparation procedures as the diagnostic specimens. Length calibrators permit determination of the size of PCR products, and defined concentrations help control the detection limit of the visualization process used. Many manufacturers offer good calibrators for length in different molecular mass ranges, so-called basepair ladders; these often permit an exact size determination of the

electrophoretically separated fragments.

Previously restricted vector DNAs also can be used as molecular mass markers, given that the digestion of vector genomes (plasmids or phages) results in defined band patterns. Because the DNA fragments are generated from the same molecule, all the fragments exist in equimolar concentrations. From the known DNA quantity added into the digestion, one can calculate the DNA quantity for each fragment as a fraction of the uncut DNA amount. Because the ethidium bromide fluorescence is proportional to the DNA quantity, and because the relative quantity of the individual bands of the calibrator is known, the concentration of PCR fragments can be judged in comparison with the fluorescence intensity of the bands of the calibrators. Many manufacturers or distributors of restriction enzymes list the precise restriction fragment lengths of vector genomes in the annexes to their manuals. Finally, basepair ladders and restricted vector calibrators can be mixed to increase the resolution of the calibration curve.

4.1.3.3. Control of blotting and of hybridization. In blot hybridizations, positive and negative controls should always be analyzed on the same membrane alongside the patient's sample reactions. This excludes misinterpretation from the use of different hybridization conditions in different reaction vessels.

4.1.3.4. Control of sequencing. Manufacturers usually supply suitable DNA amplification templates, and matching primers, as controls for their sequencing reagents. Moreover, this sequencing reaction can help control the quality of the sequencing gel electrophoresis. For analysis of mutations, the wild-type allele and samples from family members should be sequenced in parallel.

4.2. external quality control (proficiency testing)

Considering the multitude of methodological variants and diagnostic approaches, it does not appear feasible to set up external quality-assessment trials for every diagnostic problem, especially if the diseases considered are rare. This problem is particularly pertinent in smaller countries. For this reason, the Central Reference Institution of the German Society of Clinical Chemistry has started to perform its first external quality-assessment trials with two major objectives: methodological proficiency testing and application-based proficiency testing.

Methodological proficiency testing is intended to control the quality of the elementary analytical steps in molecular genetic diagnosis: the DNA/RNA preparation, the performance of the PCR method with supplied "standard primers," and the agarose gel electrophoresis. Application-based proficiency testing is at this time suitable for relatively frequent diagnostic questions, e.g., the factor V Leiden mutation in thrombophilia, caused by the resistance of clotting factor V to activated protein C; hereditary hemochromatosis; α_1-antitrypsin deficiency; mutations in apolipoproteins B100, E2, E3, and E4; and mutations in LDL receptor, ß-myosin heavy chain, and others. These will increasingly be included in nationwide quality-assessment trials in Germany. Obviously, the above-mentioned favorably complement the conventional routine panel of corresponding analytes/markers commonly determined in clinical chemistry laboratories. Continuous cell lines obtained from diseased individuals or stably transfected with genes coding for the respective gene products could be used as standardized template

sources for proficiency testing in molecular diagnostics laboratories. For rarer genetic diseases, international trials make more effective use of resources and expertise.

In conclusion, during recent years laboratory science has faced an increasing interest in molecular diagnostics and a corresponding demand for routine genetic testing. Expectations are high for two reasons: First, much is expected from molecular testing—expectations nurtured by scientific progress in, e.g., the Human Genome Project. Second, physicians have become used to a high quality of test results from the routine clinical laboratory through their day-to-day use of more-conventional laboratory markers. Although molecular tests to support clinical diagnostics will arise and prove useful over time, the issues important for the laboratories are already defined. A rapidly increasing number of laboratories are now establishing molecular technologies to use for clinical diagnosis. This results in an obvious need for standardization of both the test systems and the laboratory procedures, and efforts should be made towards this end. Particularly for the amplification-based techniques, internal laboratory procedures have to be carefully controlled. Because both the technology and its applications are in constant change and development, we emphasize that this publication is intended to recommend, not define, good laboratory practice and internal quality control at this time and to guide troubleshooting, primarily in diagnostic amplification techniques. Communication of operating procedures and scientific discussion of them is important for such guidelines to evolve. Only then will these procedures become general tools suitable for maintaining and strengthening confidence in molecular clinical testing.

Footnotes

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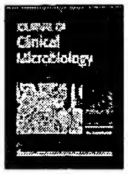

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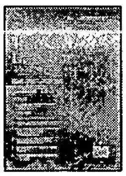

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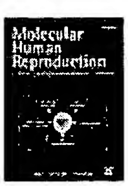

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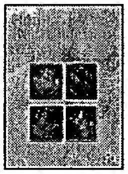

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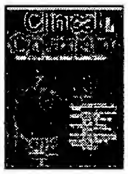

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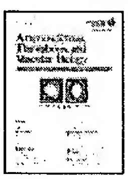

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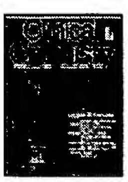

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clone from another type of organism, such as wheat. In this latter instance, the DNA sequences need not be identical but should be about 60 to 70% homologous since the conditions under which nucleic acid hybridization occurs can be manipulated to detect either partially homologous sequences or a perfectly matched duplex. For highly conserved genes, such as those for histones, the conserved sequences allow hybridization among even distantly related species.

Another hybridization technique, called *chromosome walking*, also called *overlap hybridization*, is a technique for isolating overlapping recombinants in order to "walk" from one position, usually a previously cloned and analyzed gene, to another position nearby on the chromosome. This technique, as outlined graphically in Figure 30·11, begins by isolating a terminal fragment from the starting recombinant. This terminal fragment is labelled and used as a hybridization probe for a genomic library, where it will identify all recombinants that contain it. These recombinants are then mapped with restriction endonucleases to identify those that overlap with the probe and extend into the region adjacent to the starting recombinant. The terminal fragment of this newly isolated recombinant is then used as the probe for the second "step" in the walk. This process is continued until the walk reaches the desired destination.

The nature of the destination site determines how arrival at it is identified. For example, in yeast, chromosome walking was used to move from the *LEU2* gene to a closely linked site on chromosome 3, the *centromere*. Since the centromere maintains proper segregation of chromosomes during mitosis, its presence on a recombinant was tested functionally by its ability to maintain proper segregation of plasmids in yeast cells during mitosis. In *Drosophila*, chromosome walking is routinely used to isolate genes whose function is unknown but whose physical position on the chromosome is known through detailed genetic studies. In these cases, the assay for successful completion of the walk is hybridization to the proper chromosomal band on a polytene chromosome isolated from salivary glands (Chapter 24). Drosophila geneticists have also used chromosome walking to isolate a number of very large (50 kbp and larger) genes. These genes are often interrupted by such large introns that screening a genomic library with a cDNA probe can identify two or more recombinants, each of which contains complementary sequences but neither of which is contiguous on the chromosome and neither of which includes the entire gene. Chromosome walking allows these noncontiguous recombinants to be linked, thereby establishing the complete structure of the gene.

Box 30·1 Nucleic Acid Hybridization

The specificity with which DNA and RNA molecules form stable duplex structures leads to the powerful application of this property in the technique known as *nucleic acid hybridization*. Hybridization means base pairing, and the technique is used to identify and determine the location of specific nucleic acid sequences within a larger sequence, such as a genome, recombinant DNA molecule, or mixture of RNA molecules. The stability of the duplex is directly related to the complementarity of the two nucleic acid strands. Physical conditions under which hybridization is carried out can be manipulated (referred to as altering the stringency) in order to use one nucleic acid strand as a probe for its complementary partner. Under the most stringent conditions, which include high temperature and low salt concentration, a probe sequence will hybridize only to its perfect complement. As the stringency is lowered, mismatches between the strands can be tolerated without destabilizing the

duplex. Hybridization under lower stringency is thus useful for detecting desired sequences that are partially similar but not identical, such as might be encountered when working with probe DNA and target DNA from different species. If the stringency is too low, nucleic acids will hybridize over several very short complementary regions, and specificity will be lost. A number of related techniques have been developed to apply the property of nucleic acid hybridization to screening for and isolation of specific sequences. Most of these techniques operate with a replica of the DNA of interest immobilized on a solid support, such as a nylon or nitrocellulose membrane. One of the first such methods, developed in the mid 1970's, became known colloquially as *Southern blotting,* named after its developer, Ed Southern.

In the Southern blotting procedure (Figure 1), DNA is digested with one or more restriction endonucleases and the resulting fragments separated by electrophoresis through an agarose gel. The double-stranded DNA fragments are visualized by staining, denatured in situ by soaking the gel in sodium hydroxide, and transferred to the membrane by capillary transfer in a high-concentration salt solution. These conditions allow the DNA to be retained on the filter at the point of contact between gel and filter, thus creating a replica of the gel. The DNA is then covalently bound to the filter using heat or ultraviolet light. Hybridization of probe nucleic acid (radioactively labelled DNA or RNA can be used) to the denatured DNA on the filter is carried out under the desired stringency, and after washing the excess unbound probe from the filter, the position at which specific binding occurred can be detected by autoradiography of the filter. The hybridization pattern can then be compared directly to the region of the original gel (one or a few bands) that contains the DNA sequences of interest. Southern blotting is such a powerful technique because it extends the information gained by making a restriction map of a particular piece of cloned DNA, for example, and identifies the region of that DNA that contains the sequence of interest. The Southern blotting procedure also enables nucleic acid probes to be used as diagnostic tools for many genetic disorders by probing genomic DNA from affected individuals and family members.

Following Southern's procedure, related techniques for probing DNA in situ from recombinant *E. coli* colonies (developed by M. Grunstein and D. S. Hogness) and bacteriophage plaques (developed by W. D. Benton and R. W. Davis) have been devised. In addition, J. C. Alwine, D. J. Kemp, and G. R. Stark have developed a related technique, which differs in that the gel electrophoresis fractionates not DNA but RNA. Because this technique is so similar to Southern blotting, it is known colloquially as Northern blotting. These techniques have exploited the powerful property of nucleic acid hybridization in the development of recombinant DNA technology.

B. Some Nucleic Acid Probes Are Derived from Amino Acid Sequences

Another type of information commonly available is the partial amino acid sequence of peptides generated from the protein of interest. From this information, the genetic code can be used to derive several nucleotide sequences corresponding to all possible mRNA sequences that could encode the peptide. One of these

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EXHIBIT V

of

DECLARATION

submitted under 37 C.F.R. 1.132

Maciel, S. M.; Chamberlain, C. S.; Wettermann, R. P.; and Spicer, L. J.; Dexamethasone Influences Endocrine and Ovarian Function in Dairy Cattle, Journal of Dairy Science 84:1998-2009 (2001)

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Dexamethasone Influences Endocrine and Ovarian Function in Dairy Cattle[1]

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ABSTRACT

Multiparous nonlactating Holstein cows were used to determine the effect of dexamethasone on ovarian follicular development and plasma hormone concentrations. Animals were randomly divided into two groups, control (C; n = 5) and treatment (T; n = 6), but managed as one group. Both groups were synchronized with two injections of $PGF_{2\alpha}$ (25 mg i.m.) 11 d apart. One day after ovulation (d 0) the T group received a daily injection of dexamethasone (44 μg/kg of body weight; i.m.) until the first dominant follicle stopped growing or up to d 12 postovulation. The C group received vehicle injections. Blood samples were collected daily from all cows. Concentrations of LH and FSH did not differ between the C and T cows, whereas progesterone concentrations were lower in T than in C cows from d 4 onward. Treatment × day interaction influenced plasma insulin concentrations such that T cows had insulin concentrations 2.9- to 6.0-fold those of C cows between d 2 and 9. Dexamethasone decreased IGF-I and -II concentrations from d 5 onward. Concentrations of plasma leptin and the various IGF binding proteins were not affected by dexamethasone. Total number of follicles (≥5 mm) and plasma estradiol concentrations were less in T than in C cows on d 0, 1, and 4. The growth rate of the dominant follicles and maximum diameter of the dominant and subordinate follicles were not affected by dexamethasone. The diameter of the CL was 21 to 39% larger in T than in C cows between d 6 and 10. Treatment × day interaction influenced plasma cholesterol concentrations such that cholesterol levels decreased 46.8% in T cows and 19.5% in C cows between d 0 and 10. Plasma glucose concentrations were greater in T than in C cows between d 1 and 10. In summary, dexamethasone had significant effects on metabolism without a major impact on growth of the first-wave dominant follicle. Dexamethasone-induced suppression of luteal function was associated with decreased plasma IGF-I and -II concentrations.

(**Key words:** glucocorticoids, insulin-like growth factor, follicle, ovary)

Abbreviation key: ADU = arbitrary densitometric units, **CL** = corpus luteum, **IGFBP** = IGF binding protein, **RIA** = radioimmunoassay.

INTRODUCTION

The stress-induced release of glucocorticoids is known to inhibit the immune (Sapolsky et al., 2000), reproductive (Echternkamp, 1984; Kanchev et al., 1976; Liptrap, 1993) and endocrine (Vighio and Liptrap, 1990) systems. In particular, glucocorticoids have recently been reported to stimulate leptin secretion in humans (Dagogo-Jack et al., 1997; Janssen et al., 1998; Larsson and Ahren, 1996). However, glucocorticoid effects on these systems in cattle are not well defined.

Exogenous glucocorticoids prolong the estrous cycles of cattle (Kanchev et al., 1976; Stoebel and Moberg, 1982) presumably by inhibiting LH secretion by the anterior pituitary (Li and Wagner, 1983; Padmanabhan et al., 1983) and by directly inhibiting the follicular function (Hsueh and Erickson, 1978; Schoonmaker and Erickson, 1983; Spicer and Chamberlain, 1998). Exogenous ACTH treatment in cattle, which increases endogenous cortisol secretion, induces follicular cysts (Kawate et al., 1996; Liptrap and McNally, 1976; Refsal et al., 1987). Also, in cattle, in vitro treatment of cortisol reduced the number of IGF-I binding sites in thecal cells, but had no effect on the number of granulosa cell IGF-I binding sites (Spicer and Chamberlain, 1998). In porcine granulosa cells, high concentrations of cortisol decreased the synthesis of IGF-I and IGF-I mediated steroid production (Viveiros and Liptrap, 1999). Although these studies indicate an effect of glucocorticoids at the cellular level within the ovary, little has been done to understand the systemic mechanisms by which glucocorticoids act to alter reproductive function

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in cattle, particularly its action on the endocrine system. Therefore, the objective of this study was to determine the influence of dexamethasone on plasma hormones and ovarian function of dairy cows. Dexamethasone was selected for use in this study because of its potent and specific glucocorticoid activity (Norris and Kohler, 1977; Sutanto and de Kloet, 1987) and its relatively long (i.e., 3 to 4 h) half-life (Ferguson and Hoenig, 1995; Reding et al., 1997), both of which contribute to a simplified experimental design (e.g., need of only one injection daily).

MATERIALS AND METHODS

Management of Animals and Estrus Synchronization

Eleven nonlactating multiparous Holstein cows from the Dairy Cattle Center at Oklahoma State University were randomly divided into two groups: dexamethasone-treated (n = 6) and control (n = 5) cows. Both groups were synchronized with $PGF_{2\alpha}$ (Lutalyse, Pharmacia & Upjohn, Kalamazoo, MI) using two injections of 25 mg each (i.m.), 11 d apart.

Beginning 1 d after ovulation (d 0), cows from the treatment group received dexamethasone (Azium, Schering-Plough Animal Health Corp., NJ) each day until the first dominant follicle stopped growing or up to d 12 postovulation. Each cow received 44 μg/kg of BW of dexamethasone per day (i.m.) in a volume of 1 ml/100 kg of BW per day. This dose of dexamethasone was selected based on results of previous studies that indicate similar doses of dexamethasone has biological effects in cattle (Broussard et al., 1997; Shpigel et al., 1996; Yu et al., 1997). The Control group received 1 ml/100 kg of BW per day of a vehicle solution (i.m.) containing ethanol, polyethylene glycol, and sterile-filtered millipore water at pH 4.9.

All animals were managed as one group and housed in a dry lot with access to shelter and water. This study was carried out in December 1998. During the trial, daily feeding consisted of 2 kg of grain and 7 kg of sorghum silage per head as fed, and free choice of bermudagrass hay, containing 16, 8, and 13% (% of DM) CP, respectively.

Cows were weighed daily, and BCS (1 = emaciated; 5 = obese) was recorded once on d 7 of treatment. At the beginning of the experiment, average BW for the control group was 646.3 ± 29.6 kg and 648.2 ± 27.0 kg for the treatment group. The average age of the cows (range from 3 to 9 yr) did not differ between control and treatment groups. Milk production (305 d ME) during the previous lactation averaged 11,037 ± 1,976 kg for the control animals and 10,171 ± 1869 kg for the treatment animals.

Ovarian Ultrasonography

Ovarian follicular development was monitored via daily transrectal ultrasonography using an Aloka 500 V ultrasound scanner with a 7.5 mHz probe (Corometrics Medical Systems, Inc., Wallingford, CT). Each ovary was recorded on videotape with a videocassette recorder (Mitsubishi HS-U510, Mitsubishi Electronics America, Inc., Norcross, GA). Ovarian ultrasonography was started 5 d before the initiation of treatment until 12 d after treatment.

Videotapes of the ultrasound scans were projected on a monitor, and a diagram of all follicles with diameters of ≥ 5 mm and the corpus luteum (**CL**), indicating their relative location on the ovary, were recorded for each ovary. The diameters of CL and all follicles ≥ 5 mm were calculated as the mean of the largest and the smallest diameters. The dominant follicle was determined based on the maximum diameter achieved by the largest follicle on or after d 5. To determine the growth rate of the dominant follicle, we subtracted its minimum diameter within the previous 6 d from its maximum diameter after d 5. Ovulation was determined based on the disappearance of the previous dominant follicle and the subsequent formation of a CL at the same location in the ovary.

Blood Collection and Hormone Analyses

Daily blood collection via tail venipuncture started 5 d before the beginning of the study. Blood samples were collected into 10-ml tubes containing EDTA (Monovette Sarstedt, Newton, NC) and immediately placed on ice. Samples were then centrifuged at 4°C, 1500 × *g* for 15 min, and plasma was collected and stored at −20°C until hormonal analyses were performed. For most of the hormones and metabolite assays, samples were randomly separated into two groups to be run in two assays, with samples from about the same number of cows from the control and treatment groups in each assay. All plasma samples for hormonal analyses were run in duplicate with samples thawed at room temperature.

Solid-phase radioimmunoassay (**RIA**) kits (ICN Pharmaceuticals Inc., Costa Mesa, CA) were used to measure insulin concentrations in plasma as previously described (Simpson et al., 1994). Inter- and intraassay coefficients of variation for the two insulin RIA were 8.6 and 7.6%, respectively. Sensitivity of the RIA, defined as 80% of total binding, was 0.071 ng/ml.

Double-antibody RIA was used to determine IGF-I concentrations in plasma after acid-ethanol extraction (16 h at 4°C) as previously described by Echternkamp et al. (1990). Inter- and intraassay coefficients of variation for the IGF-I RIA were 19.0 and 18.0%, respec-

tively. Sensitivity of the RIA, defined as 90% of total binding, was 0.665 ng/ml.

The concentrations of IGF-II in plasma were determined by a double-antibody RIA after extraction as previously described (Spicer et al., 1995; Stewart et al., 1996). Inter- and intraassay coefficients of variation for the IGF-II RIA were 22.7 and 7.2%, respectively. Sensitivity of the assay, defined as 80% of total binding, was 5.2 ng/ml.

A double-antibody RIA was used to determine FSH concentrations in plasma as previously described (Vizcarra et al., 1997). The intraassay coefficient of variation was 11.8%. The sensitivity of the RIA, defined as 90% of binding, was 0.03 ng/ml.

A double-antibody RIA was used to determine LH concentrations in plasma as previously described (Bishop and Wettemann, 1993). Intraassay coefficient of variation was 9.3%. Sensitivity of the RIA, defined as 90% of total binding, was 0.68 ng/ml.

Progesterone concentrations in plasma were determined with a solid-phase RIA kit (Coat-a-Count, Diagnostic Products Corporation, Los Angeles, CA) as previously described (Stewart et al., 1996). Inter- and intraassay coefficients of variation for the two progesterone RIA were 0.1 and 9.5%, respectively. Sensitivity of the RIA, defined as 95% of total binding, was 0.015 ng/ml.

Estradiol concentrations were measured in plasma samples collected on d 2, 4, 6, 8, and 10 of treatment with Serono Estradiol MAIA assay kits (Polymedco Inc., Cortlandt Manor, NY) after extraction with ethyl acetate as previously described (Vizcarra et al., 1997). Inter- and intraassay coefficients of variation for the estradiol assays were 14.5 and 9.5%, respectively. Sensitivity of the assay, defined as 90% of total binding, was 0.86 pg/ml.

Leptin concentrations in plasma were determined with a multispecies RIA kit (LINCO Research, Inc., St. Charles, MO) according to the manufacturer's recommendations with minor modifications. Briefly, samples (100 μl each) were assayed in duplicate and incubated for 24 h at 4°C with first antibody. The standard curve was modified to include 1, 2, 3, 5, 10, and 20 ng/ml of human leptin standard. On the next day, 100 μl of the ^{125}I-human leptin was added to all tubes, which were incubated for another 24 h at 4°C. On the third day, 1.0 ml of precipitating reagent (at 4°C) was added, and tubes were incubated at 4°C for 20 min. Tubes were centrifuged for 30 min at 3,000 $\times g$, supernatant from each tube was decanted, tubes were inverted one time on a paper towel to remove any excess liquid, and the precipitate was then counted in a Gamma counter. Inter- and intraassay coefficients of variation for two RIA were 6.0 and 6.6%, respectively. The sensitivity of the RIA, defined as 90% of total binding, was 0.68 ng/ml human leptin equivalent. Bovine plasma (i.e., 40, 60, 80, and 100 μl) produced curves parallel to standard curves. Recovery of mass (i.e., 1, 2, 3, and 5 ng/ml of human leptin equivalent) averaged 97 ± 6% when added to 80 μl of bovine plasma.

Glucose concentrations in plasma were determined by an enzymatic colorimetric procedure (no. 510, Sigma Chemical, Co., St. Louis, MO) as previously described (Richards et al., 1989), and the intraassay coefficient of variation was 2.75%. Sensitivity of the glucose assay, defined as the lowest value at 95% confidence interval of the lowest point on the standard curve, was 23.7 mg/dl.

Cholesterol concentrations were determined in plasma samples collected on d 0 and 10 of treatment by an enzymatic colorimetric procedure (no. 352, Sigma Chemical, Co., St. Louis, MO) as previously described (Spicer et al., 1993). The intraassay coefficient of variation was 1.26%. Sensitivity of the cholesterol assay, defined as the lowest value at 95% confidence interval of the lowest point on the standard curve, was 44.9 mg/dl.

Ligand Blots

One-dimensional SDS-PAGE was used to determine the IGF binding proteins (**IGFBP**) in plasma samples collected on d 0 and 10 of treatment as previously described (Simpson et al., 1997; Stewart et al., 1996). Briefly, 4 μl of sample was mixed with 21 μl of Laemmili sample buffer (Bio-Rad, Hercules, CA). Heat treatment was used to denature proteins (3 min at 100°C) and then samples were centrifuged at approximately 4600 $\times g$ for 3 min and added to 12-lane 12% SDS-PAGE. Samples were subjected to PAGE for about 18 to 20 h, at constant current and varying voltage. Gels were then electrophoretically transferred to nitrocellulose paper (Midwest Scientific, St. Louis, MO) for 2.5 to 3.0 h. ^{125}I-IGF II (about 15,000 cpm/0.1 ml; total volume = 6 ml) was incubated with each nitrocellulose paper at 4°C for 12 h, which was then placed on a platform shaker. The following day, Tris-buffered saline with 0.1% Tween was used to wash the nitrocellulose blots, followed by additional washings with Tris-buffered saline only. After this, nitrocellulose blots were dried and placed on an X-ray film for about 14 d at −80°C. X-ray films were then developed, and individual bands were densitometrically analyzed with Molecular Analyst (Bio-Rad) and expressed as arbitrary densitometric units (**ADU**) per 4 μl.

Statistical Analyses

This experiment was a completely randomized design with repeated measures. The data were analyzed using

the MIXED procedure (Littell et al., 1996), with sources of variation being treatment, cow within treatment (error term for treatment), day, treatment × day interaction, and residual. An autoregressive with lag equal to one model was used to model the covariance structure of the repeated measurements. If the treatment × day interaction was significant, simple effects of treatment were analyzed using the SLICE option for the LSMEANS statement (SAS, 1996). Degrees of freedom of the pooled error term were calculated by using the Satterthwait's approximation. If the treatment × day interaction was not significant, the main effects, if significant, were analyzed using the LSMEANS with the PDIFF option. For certain data (follicle and CL diameter), the maximum value during the experiment was analyzed using a completely randomized design and the GLM procedure (SAS, 1996). Data are presented as least squares means ± SEM.

RESULTS

BW and BCS

Treatment, day, and treatment × day interaction did not affect BW. Averaged over the 10-d treatment, control and dexamethasone-treated cows weighed 663.5 ± 28.4 kg and 648.7 ± 25.8 kg, respectively.

On d 7 of treatment, BCS was measured. There was no treatment effect on BCS, which averaged 3.6 ± 0.2 and 3.2 ± 0.2 in dexamethasone-treated and control cows, respectively.

Follicular Dynamics and Function

There was a trend ($P < 0.10$) for both treatment and day effects on the total number of follicles (≥ 5 mm). There was no treatment × day interaction for the total number of follicles. On d 0, 1 and 4, total numbers of ≥ 5 mm follicles were less ($P < 0.05$) in dexamethasone-treated than in control cows (Figure 1A). Total numbers of follicles decreased ($P < 0.05$) 60% between d 1 and 8 in control cows and 48% between d 1 and 8 in dexamethasone-treated cows (Figure 1A).

Dexamethasone treatment did not affect maximum diameter (15.6 ± 1.0 mm) or growth rate (1.46 ± 0.39 mm/d) of the dominant follicle. Treatment also did not affect maximum diameter (8.1 ± 1.2 mm) of the subordinate follicles. Day of treatment that the maximum diameter of the dominant follicle was achieved averaged 6.0 ± 0.6 d in Control and 6.7 ± 0.5 d in dexamethasone-treated cows and did not differ.

Luteal Development

Day ($P < 0.01$) and treatment ($P < 0.10$), but not treatment × day interaction affected the diameter of CL. Diameter of CL increased ($P < 0.05$) 52% between d 0 and 8 in control cows and 73% between d 0 and 8 in dexamethasone-treated cows (Figure 2A). Between d 6 and 10, CL diameter was 21 to 39% greater ($P < 0.10$) in dexamethasone-treated than control cows (Figure 2A). The growth rate of the CL (2.31 ± 0.41 mm/d) did not differ between control and dexamethasone-

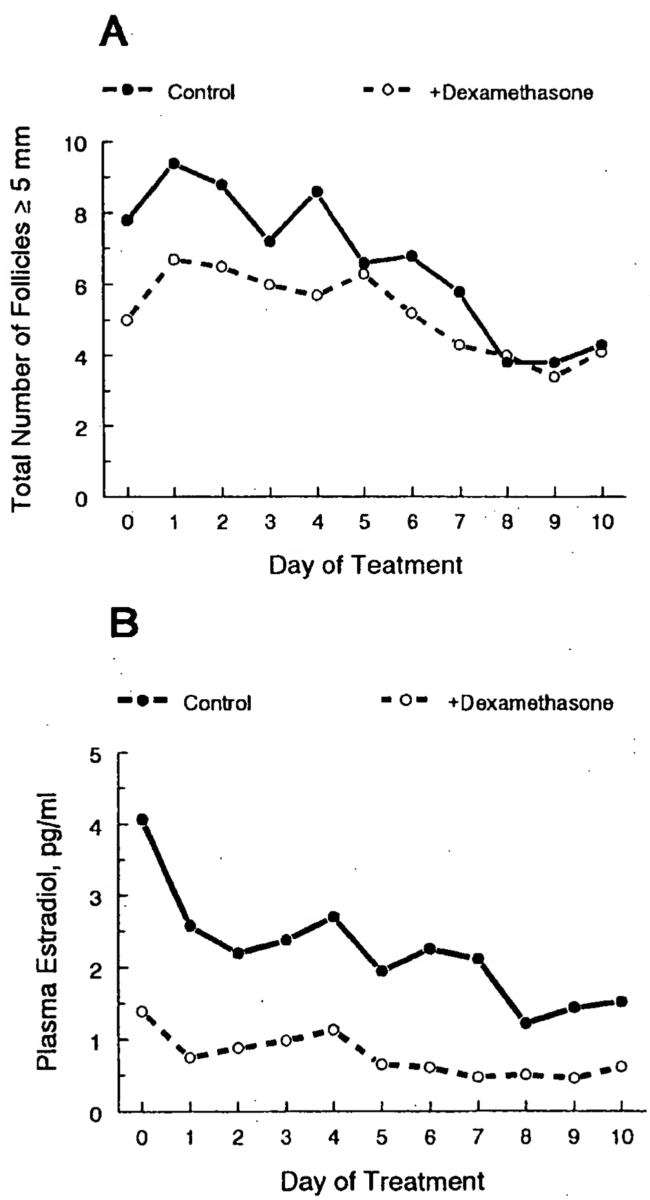

Figure 1. Changes in number of total (≥5 mm) ovarian follicles (panel A) and plasma concentrations of estradiol (panel B) in dexamethasone-treated and control cows. Pooled SE for total number of follicles averaged 0.60 per cow. Pooled SE for plasma estradiol concentrations averaged 0.22 pg/ml.

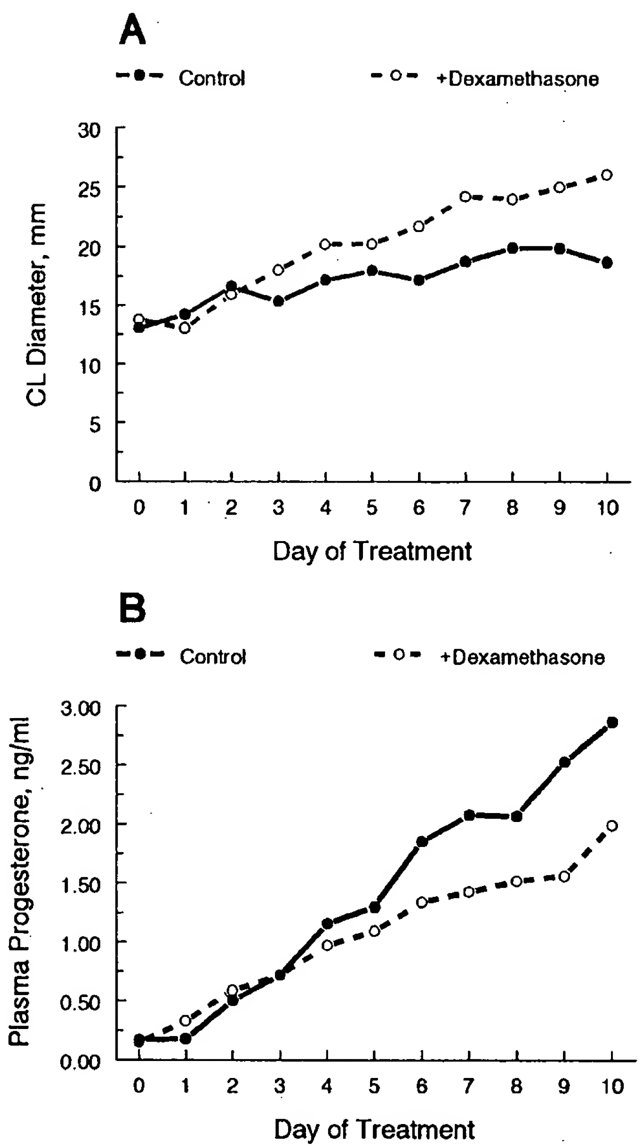

Figure 2. Changes in the diameter of corpus luteum (CL; panel A) as determined by daily rectal ultrasonography and plasma progesterone concentrations (panel B) in dexamethasone-treated and control cows. Pooled SE of CL diameter was 0.98 mm. Pooled SE for progesterone concentrations was 0.12 ng/ml.

treated cows, whereas maximum CL diameter tended ($P < 0.10$) to be greater in treated (25.8 ± 1.4 mm) than control (21.0 ± 1.6 mm) cows.

Endocrine Profiles

There was no treatment or day effect or treatment × day interaction on plasma concentrations of FSH. Concentrations of FSH averaged 0.20 ± 0.02 ng/ml and 0.19 ± 0.02 ng/ml in dexamethasone-treated and control cows, respectively.

There was no treatment effect or day effect or treatment × day interaction on plasma concentration of LH. Plasma concentrations of LH averaged 2.80 ± 0.11 ng/ml and 2.63 ± 0.11 ng/ml in dexamethasone-treated and control cows, respectively.

There was no day effect or treatment × day interaction on plasma concentrations of estradiol. However, differences in treatment groups existed ($P < 0.01$) for plasma estradiol concentrations such that plasma estradiol concentrations were greater ($P < 0.05$) in control than dexamethasone-treated cows on d 0 through 7 but not on d 8 through 10 of treatment (Figure 1B).

Day ($P < 0.001$) and treatment ($P < 0.10$) but not treatment × day interaction affected plasma progesterone (P4) concentrations. Between d 4 and 10, P4 concentrations tended to be greater ($P < 0.10$) by 15 to 40% in control versus dexamethasone-treated cows (Figure 2B). Between d 0 and 10, plasma P4 concentrations increased ($P < 0.05$) in both groups by 40 to 50% (Figure 2B).

There was a treatment × day interaction ($P < 0.05$) for plasma cholesterol concentrations. In control and treated cows, cholesterol concentrations were lower ($P < 0.01$) on d 10 (control = 90 ± 10 mg/dl vs. treated = 65 ± 8 mg/dl) versus d 0 (control = 112 ± 10 mg/dl vs., treated = 122 ± 10 mg/dl). Between d 0 and 10, plasma cholesterol concentrations decreased 46.8% in dexamethasone-treated cows versus 19.5% in control cows.

There was a treatment × day ($P < 0.0001$) interaction for plasma concentrations of glucose. From d 1 to 10 glucose concentrations were higher ($P < 0.05$) in dexamethasone-treated cows versus control cows. In treated cows, plasma glucose concentrations increased ($P < 0.05$) 72% between d 0 and 1 and then decreased ($P < 0.05$) gradually between d 1 and 10 (Figure 3A). In control cows, plasma glucose concentrations did not change between d 0 and 10 (Figure 3A).

There was a treatment × day ($P < 0.05$) interaction for plasma insulin concentrations. Between d 2 and 9, plasma insulin concentrations of dexamethasone-treated cows were 2.9- to 6.8-fold ($P < 0.05$) those of control cows (Figure 3B). Between d 0 and 3, plasma insulin concentrations increased ($P < 0.05$) in treated cows to 6.0-fold of controls. Plasma insulin concentrations remained relatively constant in control cows between d 0 and 10 (Figure 3B).

Treatment ($P < 0.05$) but not day or treatment × day interaction affected plasma IGF-I concentrations. Between d 5 and 10, plasma concentrations of IGF-I were lower ($P < 0.05$) in dexamethasone-treated cows than control cows (Figure 4A). These differences were a result of plasma IGF-I concentrations decreasing ($P <$

0.05) by 58% between d 0 and 5 in treated cows. Plasma IGF-I concentrations remained constant in control cows between d 0 and 10 (Figure 4A).

Treatment ($P < 0.01$) but not day or treatment × day interaction affected plasma IGF-II concentrations. Between d 5 and 10, plasma concentrations of IGF-II were lower ($P < 0.01$) in dexamethasone-treated cows than in control cows (Figure 4B). These differences were a result of plasma IGF-II concentrations decreasing ($P <$

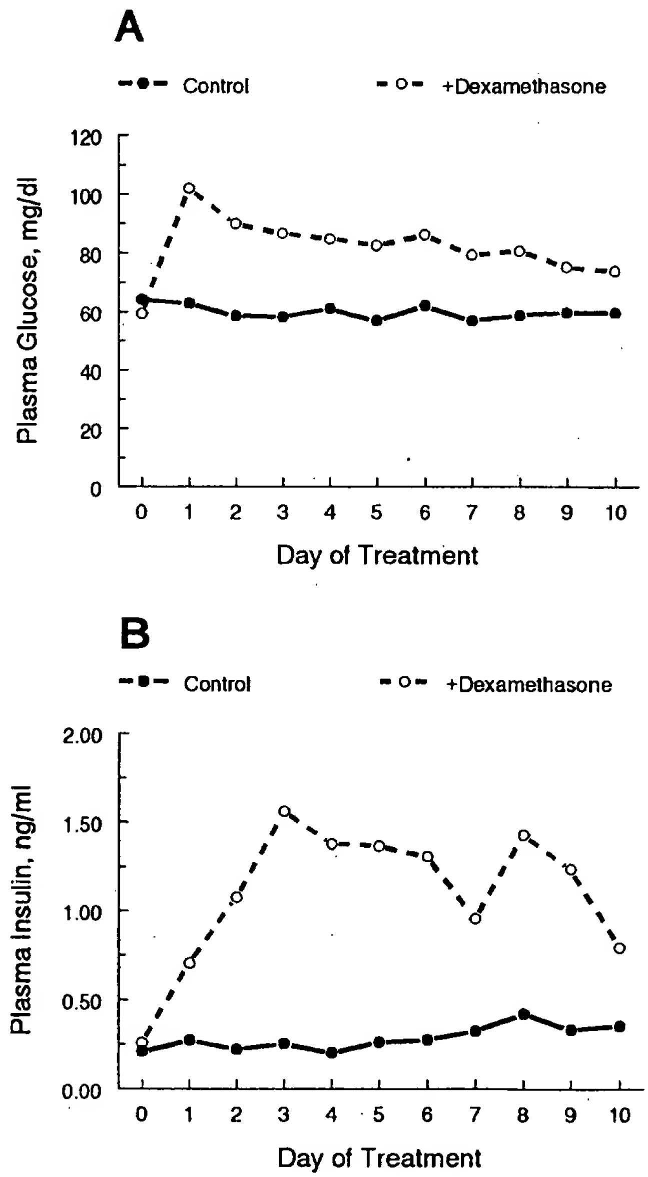

Figure 3. Changes in daily plasma glucose (panel A) and insulin (panel B) concentrations in dexamethasone-treated and control cows. Pooled SE for glucose concentrations averaged 1.61 mg/dl. Pooled SE for insulin concentrations averaged 0.08 mg/dl.

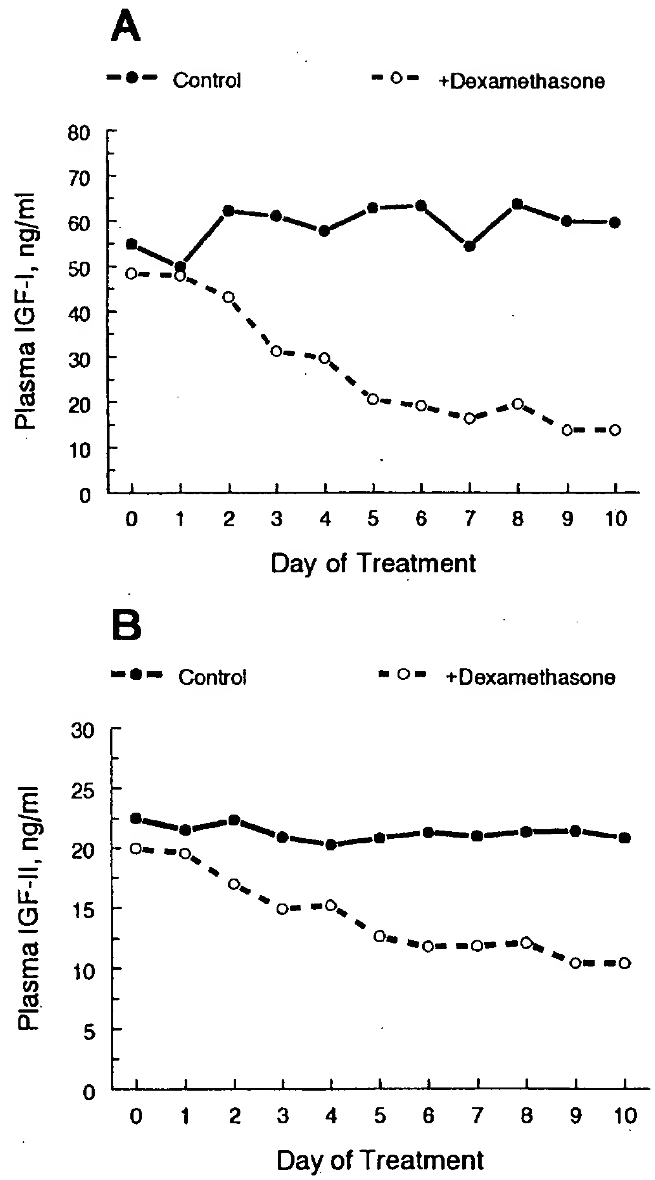

Figure 4. Changes in daily plasma concentrations of IGF-I (panel A) and IGF-II (panel B) in dexamethasone-treated and control cows. Pooled SE for IGF-I concentrations averaged 7.88 ng/ml. Pooled SE for IGF-II concentrations averaged 1.27 ng/ml.

0.05) by 35% between d 1 and 5 in treated cows. Plasma IGF-II concentrations remained constant in control cows between d 0 and 10 (Figure 4B).

Ligand blotting with [^{125}I] IGF-II revealed at least six forms of IGFBP: 34-kDa (IGFBP-2), 40- to 44-kDa (IGFBP-3), 20- to 22-kDa, 26-kDa, 28-kDa (IGFBP-4) and 30-kDa (IGFBP-5). Arbitrary densitometric units per 4 μl were used to express binding activities of the different IGFBP. Treatment, day, and treatment × day

interaction had no effect on the various IGFBP levels, except for the 26-kDa IGFBP in which a trend for day effect ($P < 0.10$) was observed. The 26-kDa IGFBP tended to decrease ($P < 0.10$) 37% in control cows and 41% in treated cows between d 0 and 10. Binding activity (ADU/4 μl) of IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5, 26-kDa IGFBP, and 20- to 22-kDa IGFBP averaged 4.00 ± 1.33, 38.2 ± 8.97, 1.27 ± 0.51, 1.16 ± 0.44, 0.77 ± 0.25, and 1.39 ± 0.22, respectively.

Treatment, day, and treatment × day interaction had no effect on plasma leptin concentrations. Plasma leptin concentrations averaged 7.78 ± 1.16 and 7.25 ± 1.27 ng/ml in dexamethasone-treated cows and control cows, respectively.

DISCUSSION

Results of the present study indicate that dexamethasone: 1) increases systemic glucose and insulin concentrations, 2) decreases plasma concentrations of IGF-I and -II without affecting IGFBP levels, 3) does not affect the growth rate of dominant follicles, 4) decreases plasma progesterone concentrations without affecting gonadotropin levels, and 5) has no effect on plasma leptin concentrations.

Similar to previous studies in rats (Dardevet et al., 1999; Marfaing et al., 1991), humans (Aaron and Tyrrell, 1994) and cattle (Andersson and Olsson, 1984; Shpigel et al., 1996), plasma insulin and glucose levels were greatly increased by dexamethasone treatment of cattle in the present study. Glucocorticoids also increase lipolysis by negating the effect of insulin (Ferguson and Hoenig, 1995; Marfaing et al., 1991) and inducing ketogenesis (Aaron and Tyrrell, 1994), which results in a diabetogenic state characterized by hyperinsulinemia and hyperglycemia, caused by insulin resistance and impaired glucose utilization (Dardevet et al., 1999; Marfaing et al., 1991). Recently, glucocorticoids were shown to protect the liver against fat accumulation in cattle via a decrease in lipid concentrations in liver and an increase in liver glycogen concentrations (Furll and Furll, 1998). However, in cultures of adipose tissue from lactating cows, dexamethasone, together with insulin, inhibits lipolysis by maintaining the intracellular G-protein signaling system (Lanna and Bauman, 1999). Thus, the dexamethasone-induced increase in plasma glucose and insulin obtained in our study agrees with the results previously described and confirms glucocorticoid effects on metabolism in dairy cattle.

For the first time in cattle, we report a suppressing effect of dexamethasone on plasma IGF-I concentrations, which is consistent with previous studies in rats (Ohyama et al., 1997) and chickens (Leili and Scanes, 1998). Increases in hepatic IGF-I mRNA induced by GH are also inhibited by dexamethasone in vitro (Beauloye et al., 1999). In rats, glucocorticoid treatment decreased IGF-I mRNA in neuronal cells (Adamo et al., 1988), bone cells (McCarthy et al., 1990), and muscle tissue (Gayan-Ramirez et al., 1999). Because GH receptor mRNA is also suppressed by dexamethasone (Beauloye et al., 1999), the inhibitory action of dexamethasone on IGF-I secretion is likely at the level of the liver as well as the anterior pituitary. In agreement with this, Elsasser et al. (1997) suggests that chronic high concentrations of glucocorticoids decrease plasma GH and IGF-I concentrations in cattle.

For the first time in ruminants, we report a suppressing effect of dexamethasone on plasma IGF-II concentrations, which is consistent with a previous report in mice (Rooman et al., 1999). Because IGF-II mRNA expression is decreased by corticosteroid in the gastrocnemius and diaphragm of rats (Gayan-Ramirez et al., 1999), glucocorticoids may directly inhibit hepatic IGF-II gene expression in cattle.

Dexamethasone did not have an effect on plasma IGFBP levels in the present study, a finding not previously reported for cattle. In rats, production of IGFBP-2 is enhanced by dexamethasone, while IGFBP-3 is reduced, thus inhibiting the transport of IGFs (Villafuerte et al., 1995). These IGFBP, with molecular masses between 17 and 43 kDa, regulate the action of IGF by competing with the IGF receptor for IGF binding and thus inhibiting IGF-I activity and bioavailability (Jones and Clemmons, 1995; Spicer and Echternkamp, 1995). According to their molecular masses characterized for cattle (Funston et al., 1996; Spicer and Echternkamp, 1995), we identified IGFBP-2 (34 kDa), IGFBP-3 (40–44 kDa), IGFBP-5 (30 kDa), and IGFBP-4 (28 kDa). The IGFBP with molecular masses of 20 and 22 kDa are likely deglycosilated forms of IGFBP-4 (Carr et al., 1994; Funston et al., 1996). The 26-kDa IGFBP was the least prevalent IGFBP and tended to decrease with day of cycle regardless of treatment. The identity of the 26-kDa IGFBP is uncertain but, based on its molecular mass, is likely IGFBP-1 (Rechler, 1993). Because IGF-I and -II levels were reduced and IGFBP were not affected by dexamethasone treatment, we can conclude that dexamethasone directly reduces the amount of bioavailable IGF-I and -II in plasma of cattle.

We observed no effect of dexamethasone on FSH or LH secretion, which is in general disagreement with previous in vitro studies in cattle (Li and Wagner, 1983; Padmanabhan et al., 1983) and rats (Suter and Schwartz, 1985), and in vivo studies in cattle (Echternkamp, 1984), rats (Tohei and Kogo, 1999) and sheep (Daley et al., 2000); however, most of these reports refer to the need of high doses of glucocorticoids to alter

gonadotropin secretion. Although 10 to 20 ng/ml of systemic cortisol concentrations do not affect LH, a 10- to 20-fold increase in plasma cortisol, due to intensive stress, suppresses tonic LH by mainly reducing the pulsatile releases of LH (Echternkamp, 1984). Blood samples were collected too infrequently (i.e., daily) to evaluate an effect on LH pulsatility in the present study. However, in agreement with the present study, an increase in plasma glucocorticoids had no effect on plasma LH concentrations, with samples collected every 20 min for 8 h in dairy cows (Hockett et al., 2000) or every 15 min for 12 h in cows with regular estrous cycles (Vighio and Liptrap, 1990). Similarly, dexamethasone had no effect on LH secretion in ewes (Phillips and Clarke, 1990). In rats, dexamethasone treatment (60 to 600 ng/ml) stimulates FSH synthesis and secretion in vitro (Suter and Schwartz, 1985) and in vivo (Tohei and Kogo, 1999). The stimulatory effect of glucocorticoids on basal secretion of FSH might mean a positive and specific role for glucocorticoids in reproductive function (Mahesh and Brann, 1998; Suter and Schwartz, 1985). Treatment with ACTH (3 mg, i.m.) for 14 d during the late luteal phase in cattle suppressed plasma FSH concentrations and the preovulatory surge of FSH (Kawate et al., 1996). However, dexamethasone (2 mg/d) had no effect on FSH secretion in ewes (Phillips and Clarke, 1990). The secondary surge of FSH in women, which is the regulator of follicle development, is corticosteroid-dependent, and this might explain why glucocorticoids have been successfully used to treat ovulatory failure in women (Mahesh and Brann, 1998). Based on these studies, it seems that the dexamethasone dosage or period of treatment used in our study was not high enough or long enough to alter either LH or FSH secretion.

On d 0, 1, and 4, dexamethasone-treated cows tended to have fewer total follicles than the control cows. Similarly, plasma estradiol concentrations were lower in dexamethasone-treated versus control cows on d 0 to 7 of treatment when the dominant follicle was growing. However, differences existing on d 0 could not be attributed to treatment per se. More important perhaps was the observation that growth rate and maximum size of the dominant follicle was not affected by dexamethasone in the present study nor were there differences in plasma estradiol concentrations after 8 or 10 d of treatment, when the first-wave dominant follicle had stopped growing. Dexamethasone treatment (10 mg/d) in diestrous cows between d 13 and 22 decreased estradiol concentrations throughout the estrous cycle, never rising above 3 pg/ml (Broussard et al., 1997). In vitro, cortisol at 100 ng/ml has no effect on aromatase activity of small-follicle granulosa cells but inhibits aromatase activity of large-follicle granulosa cells by 24% (Spicer and Chamberlain, 1998). Because plasma estradiol levels were lower on d 2, 4, and 6 but did not differ on d 8 and 10 of treatment, the present and previous studies indicate that dexamethasone may have had a direct role in inhibiting estradiol production by developing follicles. However, differences in numbers of follicles and estradiol at the start of treatment confound that conclusion.

In our study we also found that the dexamethasone-treated cows had a larger CL and lower systemic progesterone concentrations. Previous studies indicate that dexamethasone treatment in cows extended luteal function due to delayed or impairment of preovulatory follicular development (Broussard et al., 1997; Kanchev et al., 1976). Comparing different breeds (Brahman, Senepol, and Angus) of cattle, larger luteal growth in the Brahman and Senepol cows are associated with greater amounts of IGF-I in plasma (Alvarez et al., 2000), and in dairy cows decreased luteal progesterone secretion is related with a reduction in plasma IGF-I (Spicer et al., 1990). Regarding its ovarian functions in livestock species, IGF-I and -II stimulate the delivery of cholesterol to the P450scc enzyme complex, the rate-limiting step in progesterone biosynthesis (Spicer and Echternkamp, 1995; Veldhuis and Gwynne, 1989). Also, IGF-I and -II stimulate progesterone release by luteal cells and tissues in vitro (Liebermann et al., 1996; Sauerwein et al., 1992). Thus, reduced IGF-I and -II concentrations in dexamethasone-treated cows of the present study may have contributed to the decrease in plasma progesterone levels. Why dexamethasone treatment increased CL size but reduced systemic progesterone concentrations in the present study is unknown. Perhaps dexamethasone treatment induced hepatic enzymes involved with steroid hormone metabolism and clearance, and thus, increased progesterone clearance from the systemic circulation. In support of the latter suggestion, previous studies indicate that dexamethasone induces cytochrome P450 3A in rats (Lee and Lee, 1999; Wright and Paine, 1994) and progesterone-6-β-hydroxylase in chick embryo liver (Kimmett et al., 1996). Further work will be needed to clarify these possibilities.

A greater decrease in plasma cholesterol concentrations between d 0 and 10 was observed in dexamethasone-treated versus control cows. In mammals, the primary source of systemic cholesterol comes from the diet or from liver synthesis. The required cholesterol for steroidogenesis is obtained from circulating serum low- and high-density lipoproteins and through de novo cholesterol synthesis mediated by hepatic 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (Savion et al., 1982; Veldhuis and Gwynne, 1989). Both processes, utilization of low density lipoprotein and de novo synthesis of cholesterol, are stimulated by IGF-I (Spicer and Echt-

ernkamp, 1995; Spicer et al., 1996; Veldhuis and Gwynne, 1989). Because plasma cholesterol was reduced 46.8% in dexamethasone-treated cows versus 19.5% in the control cows, it might be that cholesterol synthesis was decreased due to dexamethasone suppressing IGF-induced hepatic 3-hydroxy-3-methyl-glutaryl coenzyme A reductase at the level of the liver. This greater decrease in plasma cholesterol and lower plasma IGF-I and -II levels in dexamethasone-treated versus control cows may have contributed to lower the plasma progesterone concentrations observed in treated cows.

We found that the Linco 'multi-species' RIA kit gave reproducible and valid assessment of relative "immunoreactive" leptin concentrations in plasma of cattle. Because recombinant bovine leptin is not commercially available, no exact quantification or comparison could be made in terms of the concentration of "bovine" leptin in plasma of cows in the present study. Recent reports indicate that the 'multi-species' RIA kit of Linco Research Inc. may be limited in usefulness for measuring leptin in blood of sheep (Blache et al., 2000; Delavaud et al., 2000; Ehrhardt et al., 2000) and cattle (Amstalden et al., 2000; Ehrhardt et al., 2000) without providing substantitive evidence. Once recombinant bovine leptin becomes commercially available, further clarification on the usefulness of the 'multi-species' RIA kit will likely ensue. In contrast to studies conducted in humans (Dagogo-Jack et al., 1997; Janssen et al., 1998; Larsson and Ahren, 1996), we found no effect of dexamethasone on leptin secretion in cattle. In bovine adipose tissue culture, only high concentrations of dexamethasone (100 n*M*) have stimulatory effects on leptin mRNA levels (Housecknecht et al., 2000), whereas in cultured human adipocytes, 50 n*M* of dexamethasone stimulates leptin secretion (Halleux et al., 1998). Thus, bovine adipocytes may be less responsive than human adipocytes to the stimulatory effect of glucocorticoids on leptin production and the dose of dexamethasone used was probably not high enough (44 μg/kg of BW) to affect plasma leptin levels. Further research will be needed to clarify these possibilities.

Previously, two types of corticosteroid receptors have been identifed: type I that are more related to corticosterone activity (F), and type II, which are associated with cortisol activity (B) and represent the classical glucocorticoid receptor system (Sutanto and de Kloet, 1987). Studies reporting the relative binding affinities (IC_{50}) of cortisol and dexamethasone for type I and II receptors in the hamster found that dexamethasone is 75% and 64-fold as effective as cortisol for binding to the type II and I receptor, respectively (Sutanto and de Kloet, 1987). Another study reported that dexamethasone was 1.4-fold more potent than cortisol for inhibiting specifically bound [^{3}H] cortisol (Norris and Kohler, 1977). We administered on average a total of 28.8 mg of dexamethasone per day, which would have resulted in a dexamethasone concentration of 880 ng/ml at a maximum and 0.88 ng/ml still present in bovine plasma at 24 h after treatment based on the clearance rate of dexamethasone in humans (Cassidy et al., 1998) and cattle (Reding et al., 1997). The estimated half-life of dexamethasone (AZIUM solution) following intramuscular administration is about 3 to 4 h (Ferguson and Hoeing, 1996). Based on the reported affinities of dexamethasone for the glucocorticoid receptor (Norris and Kohler, 1977; Sutanto and de Kloet, 1987), the "cortisol equivalent" dose achieved systemically in each cow would have ranged between 1 and 200 ng/ml during a 24-h period. In female cattle, physiological levels of cortisol in the blood range between 3 and 15 ng/ml (Garverick et al., 1971; Swanson et al., 1972; Roussel et al., 1983), and under stressful conditions cortisol concentrations vary between 10 and 100 ng/ml (Echternkamp, 1984). Therefore, the daily dose of dexamethasone used in the present study closely mimicked physiological stress-induced releases of glucocorticoids.

CONCLUSIONS

This was the first comprehensive in vivo study on the effects of an exogenous glucocorticoid on metabolic and reproductive functions of dairy cows. Suppression of luteal function by short-term (10 d) glucocorticoid treatment was associated with decreased concentrations of cholesterol, IGF-I and -II in plasma. The mechanism by which glucocorticoids regulate IGF-I and -II secretion in cattle will require further study. Dexamethasone also dramatically increased plasma glucose and insulin levels without affecting plasma gonadotropin levels or growth of the dominant follicle. Thus, short-term (i.e., 10 d) increases in systemic levels of glucocorticoids have little direct impact of follicular function but may have a negative impact on luteal function in dairy cattle.

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COMMENTS

Dexamethasone Increases Leptin Expression in Humans *in Vivo*

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ABSTRACT

The effect of 2 days of oral dexamethasone administration (0.75 mg twice daily) on leptin expression in healthy volunteers was tested. Dexamethasone increased the relative abundance of leptin messenger RNA in abdominal and gluteal adipose tissues by approximately 70% ($P < 0.05$). Dexamethasone also significantly increased serum leptin (+ 80%) and insulin concentration (+ 83%) but did not affect serum glucose. We conclude that a hypercortisolemic/hyperinsulinemic state up-regulates leptin expression at the messenger RNA level in humans. (*J Clin Endocrinol Metab* **82:** 1635–1637, 1997)

THE ADIPOCYTE hormone leptin is thought to serve as a signal to the central nervous system reflecting the status of fat stores. Serum levels of leptin and adipocyte leptin messenger RNA (mRNA) levels are clearly increased in obesity (1, 2). However, the factors regulating leptin production in obesity are not yet understood. Hyperinsulinemia is a logical candidate, and indeed, insulin appears to be a long-term regulator of leptin expression in humans (3, 4). Obesity is also associated with increased cortisol turnover (5, 6). In combination with insulin, glucocorticoids increase the expression of genes important in regulating lipid deposition in the adipocyte, including lipoprotein lipase (LPL) (7, 8). Glucocorticoids also increase leptin expression in rodents, *in vivo* and *in vitro* (9–11).

The primary objective of the present study was to determine the effects of *in vivo* administration of the synthetic glucocorticoid dexamethasone on levels of adipose tissue leptin mRNA and serum leptin in humans. A dose of dexamethasone was chosen to produce a high physiological level of glucocorticoid activity. Because glucocorticosteroids induce insulin resistance (12–14), we assessed effects of dexamethasone administration on serum insulin levels. Additionally, to gain insight into metabolic effects of elevated glucocorticoids in humans, expression of lipoprotein lipase (LPL) was determined.

Materials and Methods

Subjects and procedures

Subject characteristics are shown in Table 1. Six healthy, nonsmoking, weight-stable, overweight volunteers were subjects in Study 1. They were free of metabolic disease and not taking medications (except for one subject on a stable dose of Prozac whose results did not differ from the rest of the group). Subjects reported for medical screening and baseline determinations of weight and height after a 12 h overnight fast. Blood was drawn for determination of serum insulin, cortisol, glucose, and leptin. Subcutaneous adipose tissue was aspirated from the abdominal and gluteal regions under local lidocaine anesthesia, as previously decribed (15). Subjects were instructed to ingest tablets containing a total of 1.5 mg dexamethasone per day (0.75 mg, 2 × daily with breakfast and dinner) on two consecutive days. They returned on the following morning after an overnight fast for a second blood sample and adipose tissue aspirations (on the contralateral side of the body from the first sample). Subjects were also instructed to keep food records on one control day (a weekday without the drug) and during the treament period. They were not given any instructions on diet composition or daily intake. Unfortunately, serum samples for all but one subject in Study 1 were not available for insulin, glucose, or leptin determinations. Thus, in Study 2, eight healthy subjects were recruited for serum sampling before and after dexamethasone administration using the same protocol, excluding the adipose tissue aspirations. One male subject in Study 2 was a light smoker and continued this behavior throughout the study. His results were included as they did not differ from the rest of the group. These studies were approved by the Institutional Review Board of the University of Medicine and Dentistry of New Jersey and Rutgers University.

Adipose tissue (1 gm) was immediately frozen for later extraction of RNA and Northern blotting, as previously described (7). A complimentary DNA for human leptin was obtained from Dr. J.M. Friedman (The Rockefeller University, New York, NY). A 1 kb *EcoRI* fragment was labeled with ^{32}P-dCTP for use as a probe. RNA extraction and Northern analysis was carried out as previously described (7). The intensity of the autoradiographic images were quantified after scanning using UN-SCAN-IT software (Silk Scientific, Orem, UT). In a subset of subjects, it was determined that leptin expression was restricted to the adipose cell fraction of adipose tissue (data not shown). Assays of lipoprotein lipase activity (7) and adipose cell sizing after osmium fixation (16) were carried out as previously described. Serum samples were frozen for radioimmunoassay of insulin (17), cortisol (kit from Diagnostic Systems Laboratories, Inc, Webster, TX), and leptin (kit from Linco, St. Charles, MO). Serum glucose was determined using a Beckman glucose analyzer.

Statistics

After log-transformation of the data, the effect of dexamethasone on serum parameters, and the relative abundance of mRNAs (ratio to 28S ribosomal RNA) were determined by two-tailed (unless indicated) paired *t*-tests (Excel, Microsoft). Probabilities less than 0.05 were considered statistically significant.

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TABLE 1. Subject characteristics

	Mean ± SEM	Range
Study 1 (3F, 3M)		
BMI (kg/m^2)	29.3 ± 1.0	26.6–32.0
Age (yr)	29 ± 2	23–35
Study 2 (5F, 4M)		
BMI (kg/m^2)	26.7 ± 1.5	22.2–34.8
Age (yr)	31 ± 2	25–44

TABLE 2. Serum values before and after dexamethasone administration

	Control	Dexamethasone
Cortisol (μg/dL)[a]	14. ± 2	<1.0[b]
Glucose (mg/dL)[c]	100 ± 3	96 ± 4
Insulin (μU/mL)[c]	29 ± 14	42 ± 14[b]

[a] n = 14 (Study 1 and 2).
[b] $P < 0.05$ *vs.* control.
[c] n = 9 (Study 2).

Results

As indicated by the mean body mass indices (BMI), subjects were on average overweight (Table 1). The range of values, however, was wider in Study 2 than Study 1. As expected, dexamethasone (dex) administration suppressed endogenous cortisol in all subjects (Table 2), indicating good compliance with the protocol. Serum insulin levels also increased by an average of 83 ± 29% over control values, but serum glucose did not change (Table 2).

Leptin mRNA levels

Two days of dexamethasone treatment increased the relative abundance of leptin mRNA by 70 ± 28% in the abdominal depot and 68 ± 25% in the gluteal depot (n = 6, $P < 0.05$). A typical autoradiograph of a Northern blot and a densitometric quantitation of leptin/28S are illustrated in Fig. 1.

The relative abundance of LPL mRNA also increased moderately (+33 ± 7%) in the gluteal depot after dexamethasone (LPL/28S ratio: 0.91 ± 0.48 (before dex) *vs.* 1.2 ± 0.66 (after dex), (n = 6, $P < 0.01$), but not significantly in the abdominal depot (although in the females the effect was consistent (+ 45 ± 14%, $P < 0.05$ using one-tailed *t*-test, n = 3). LPL enzymatic activity did not significantly increase (data not shown).

Serum leptin

In Study 2, serum leptin levels increased by 80 ± 17%, n = 9, after two days of dexamethasone ($P < 0.005$; Fig. 2).

Discussion

We have demonstrated that induction of mild hypercortisolemia for two days increased leptin mRNA levels by 70% in both gluteal and abdominal subcutaneous adipose tissues and increased plasma leptin levels by 80%. Dexamethasone also produced an 83% increase in plasma insulin levels. Thus, we conclude that a hypercortisolemic, hyperinsulinemic state increases leptin expression in humans. The parallel rise in leptin mRNA levels and serum leptin levels after dexamethasone administration strongly suggests that the effect is mediated at the mRNA level.

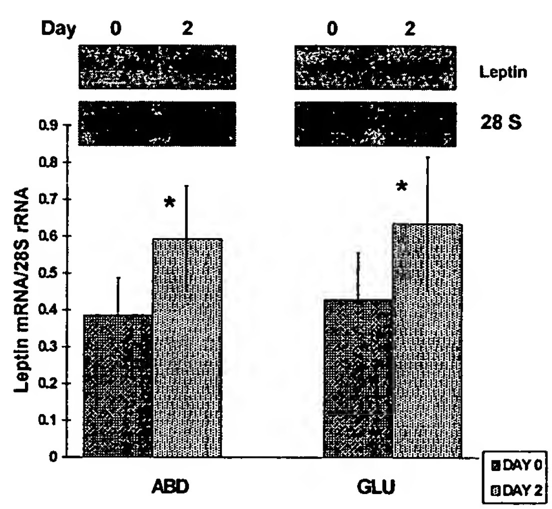

FIG. 1. Effect of dexamethasone on leptin mRNA levels. *Bars* represent means ± SEM of the 6 subjects in Study 1 before (day 0) and after (day 2) dexamethasone administration for 48 h. Representative autoradiograph of a Northern blot shown above the bars. * $P < 0.05$.

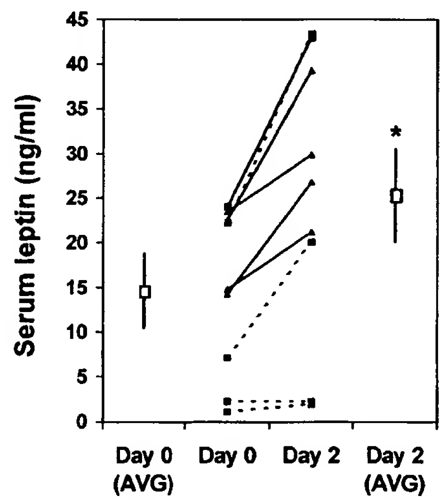

FIG. 2. Effect of dexamethasone administration on serum leptin levels. *Lines* connect individual values before and after dexamethasone administration in men (----) and women (———). *Square symbols* represent the mean ± SEM (n = 9). * $P < 0.01$.

The stimulatory effect of glucocorticoid on serum insulin that we observed is consistent with several previous reports (12–14), although one study using methylprednisolone found no effect (18). A recent study showed that a dose of 2 mg/day dexamethasone did not influence glucose tolerance whereas higher, pharmacological doses did (12). Thus, it is likely that we were successful in inducing a moderately hypercortisolemic state, in which hyperinsulinemia compensated for mild peripheral insulin resistance.

It is possible that the doubling of fasting insulin induced by dexamethasone contributes to the increase in leptin expression that we observed. Kolaczynski *et al.* (3) reported that serum leptin levels were not increased until the final 48–72 h period of a hyperinsulinemic-euglycemic clamp in lean subjects. Preliminary observations in two lean female sub-

jects showed that levels of serum leptin and insulin had already doubled after 30 h of dexamethasone administration. Additional studies of the time course of glucocorticoid and insulin effects during clamped conditions are needed to resolve whether dexamethasone stimulates leptin without concomitant hyperinsulinemia, how rapidly this effect occurs, and whether there are gender or obesity-related differences in responsiveness to these hormones.

The permissive or synergistic effects of insulin and glucocorticoids in regulating leptin mRNA expression are supported by our recent studies of human adipose tissue from obese subjects in organ culture (19). The combination of insulin and dexamethasone, but not insulin or dexamethasone alone, consistently stimulated leptin mRNA relative abundance in subcutaneous adipose tissue.

Gender-specific depot differences in the expression of glucocorticoid receptor mRNA levels have been observed between abdominal (abd) and gluteal (glu) adipose tissues (men: abd < glu; women abd > glut) (20). Thus, we expected a depot difference in the expression of leptin and responsiveness to glucocorticoids. However, both depots responded similarly.

In addition to increasing leptin expression, dexamethasone administration also increased the expression of LPL mRNA in the gluteal depot. The moderate increase in mRNA level was not accompanied by a statistically significant rise in LPL activity, as we would have predicted from our *in vitro* studies (7). The time course of induction of LPL may lag behind the induction of message.

Increases in serum leptin would be expected to lead to a suppression of food intake. However, we did not observe any effects of dexamethasone treatment on spontaneous food intake, as indicated from diet records (unpublished observation). A recent study by Tataranni *et al.* (13) found an increase in food intake as a consequence of administration of the glucocorticoid methylprednisolone. However, a preliminary report indicated that they found no change in serum leptin after 4 days of treatment (21). Discrepancies with the present study may be explained by the higher dose (40 mg methylprednislone is equivalent to 7.5 mg dexamethasone), time-dependent effects, the use of exclusively lean male subjects, or more accurate measures of food intake in an inpatient study. However, it is possible that the rise in leptin that occured at the lower dose of glucocorticoids in our subjects may have offset any stimulatory effects on appetite (6, 21). Alternatively, we speculate that glucocorticoids may decrease sensitivity to leptin's inhibitory effects on food intake, leading eventually to an increased body weight "set point". Whether this mechanism contributes to the obesity that occurs clinically in hypercortisolemic patients remains to be determined. Because the balance between insulin and glucocorticoid is thought to be crucial in regulating energy balance (6), in future studies it will be important to undertake careful measurements of food intake during administration of varying doses of glucocorticosteroids in lean compared with obese men and women.

In conclusion, a moderate elevation in serum insulin and glucocorticoids increased leptin mRNA levels in subcutaneous adipose tissues and serum leptin levels in humans within 48 h. The concommitant hyperinsulinemia and increased cortisol turnover associated with the obese state may be important modulators of the degree of hyperleptinemia.

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Note Added in Proof

After submission of this manuscript, Larsson and Ahren (J Clin Endocrinol Metab 81:4428–4432, 1996) also reported that dexamethasone (3 mg/day for 48 h) increased plasma leptin.

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EXHIBIT Q

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Nanda, I., Kondo, M., Hornung, U., Asakawa, S., Winkler, C., Shimizu, A., Shan, Z., Haaf, T., Shimizu, N., Shima, A., Schmid, M., and Schartl, M., A Duplicated Copy of DMRT1 in the Sex-Determining Region of the Y Chromosome of the Medaka, *Oryzias Latipes*; Proc Natl Acad Sci USA; 2002 Sept. 3; 99(18): 11778-11783

A duplicated copy of *DMRT1* in the sex-determining region of the Y chromosome of the medaka, *Oryzias latipes*

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The genes that determine the development of the male or female sex are known in *Caenorhabditis elegans*, *Drosophila*, and most mammals. In many other organisms the existence of sex-determining factors has been shown by genetic evidence but the genes are unknown. We have found that in the fish medaka the Y chromosome-specific region spans only about 280 kb. It contains a duplicated copy of the autosomal *DMRT1* gene, named *DMRT1Y*. This is the only functional gene in this chromosome segment and maps precisely to the male sex-determining locus. The gene is expressed during male embryonic and larval development and in the Sertoli cells of the adult testes. These features make *DMRT1Y* a candidate for the medaka male sex-determining gene.

The overwhelming majority of multicellular animal species occur as both sexes, and in many cases the decision of whether an organism becomes a male or a female is determined by the genome. In most mammals, several flies, and the worm *Caenorhabditis elegans* the cascades of sex-determining genes are reasonably well understood. However, the master regulator encoded by the sex-determining locus (*SD*) on the Y chromosome of most mammalian species, *SRY*, is not functioning like that in some mammals (1). In nonmammalian species, which also have a XX/XY sex-determination system, *SRY* is not present at all. Neither the *sxl* gene of *Drosophila* nor *xol* of *C. elegans*, the genes at the top of the sex-determination cascade in these organisms, are functioning in the same way in more distantly related species (for review see ref. 2). This finding indicates that on the molecular level another dimension of diversity is added to the complex situation of multiple genetic systems for sex determination. In between worms and flies on the one side and mammals on the other side, there is a large gap in our knowledge about sex-determination genes.

Fishes are an attractive group of organisms for studying the evolution of sex determination because members of this class exemplify a broad range of various types of sex determination from hermaphroditism to gonochorism and from environmental to genetic sex determination (for review see refs. 3 and 4). The structure and expression of genes involved in sex determination and differentiation can be compared in species, which exhibit either similar or divergent sex-determination systems. Unfortunately, in both main fish models, the pufferfish (*Takifugu rubripes*) and the zebrafish (*Danio rerio*), no information exists on the mode of sex determination, the potential presence of sex chromosomes, and the process of sex differentiation.

The situation for a molecular analysis of sex determination is much more favorable in another fish model, the medaka (*Oryzias latipes*) (for review see ref. 5). Medaka has a XX/XY sex determination system like mammals. Male and female medakas are easily distinguished by a number of secondary sex characters (see ref. 6). A linkage map of the sex chromosomes with several molecular and phenotypic markers is available (7, 8). Especially useful is the *quart* strain, where the sex chromosomes express different alleles of the *lf* pigment marker. The presence of leucophores in the male and their absence in the female allow differentiation of both sexes as early as at 2–3 days of embryonic development (9).

From the genes known to be involved in sex determination/differentiation the *DMRT* genes are of special interest because of their widespread distribution. They form a family of genes, which share a highly conserved DNA-binding domain (10), the DM domain. *DMRT1* is a candidate downstream sex-determination gene in mammals and appears to be involved in a certain type of XY sex reversal in humans (11–13). It is conserved in a wide range of animals with diverse sex-determining mechanisms, including *C. elegans*, *Drosophila*, fish, reptiles, birds, and mammals (14–19).

We have investigated a potential role for *DMRT* genes in sex determination of medaka and found that a duplicated copy of *DMRT1* is present at the male *SD* and shows all features of a sex-determining gene.

Materials and Methods

Medaka Fish. All experimental animals were from inbred lines of Northern (Kaga, HNI) and Southern medakas (i-3, quart, HB32C, SOK) except for the Carbio strain, which is an outbred strain derived from the Southern population of medaka.

Cloning and Sequence Analysis. To obtain the Y-chromosomal *DMRT* gene the male-specific band from Southern blots of genomic DNA was excised and cloned. Briefly, the 3.3-kb male-specific *Eco*RI fragment from medaka (strain Carbio) was cloned by excising the 3- to 4-kb *Eco*RI fragments from a preparative 0.8% agarose gel. The DNA was ligated into λZapII *Eco*RI arms (Stratagene) and packaged *in vitro*. The resulting subgenomic library was screened with the 4-kb *Eco*RI fragment from medaka cosmid 73K2481, which contains exons 1–3 of the autosomal *DMRT1* gene.

Filters of arrayed medaka genomic cosmid libraries (nos. 73 and 74) were obtained from the Resource Center of the German Human Genome Project (Berlin) and screened with the human *DMRT1* cDNA under conditions of low stringency (hybridization: 35% formamide, 42°C; washing: 1× SSC/1% SDS, 63°C). Positive clones were initially characterized and arranged in groups by restriction fragment analysis and Southern blotting.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviation: BAC, bacterial artificial chromosome.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AY129240 and AY129241).

†I.N. and M.K. contributed equally to this work.

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Restriction fragments cross-hybridizing with the human *DMRT1* probe were subcloned and sequenced.

For isolation of a medaka *DMRT1Y* cDNA, total RNA was isolated from 6-day-old embryos (strain Carbio). Reverse transcription was done with oligo(dT)$_{12-18}$ and Superscript II reverse transcriptase following the supplier's protocol (GIBCO/BRL). For PCR from 3 μl of the reverse transcription reaction, primers based on the genomic sequence of *DMRT1Y* (DMTYh: TCT GCT GAG CTC CCC GGG; DMTYi: GCC TCG CAG CTT CTC A) were used. The PCR was run for 35 cycles at an annealing temperature of 62°C. The sequence of *DMRT1Y* is deposited under GenBank accession no. AY129240.

Bacterial Artificial Chromosome (BAC) Isolation and Analysis. A genomic BAC library of the HNI strain with an average insert size of 160 kb was constructed (20). BAC clones containing the DM domain sequences were screened by colony hybridization under low stringency with the PCR fragment of the DM domain of medaka *DMRT4* (20). The clones containing *DMRT1Y* were selected by PCR screening with a specific primer set (DMTk: CAA CTT TGT CCA AAC TCT GA; DMTl: AAC TAA TTC ATC CCC ATT CC). The contig was extended by using PCR end fragments as probes, and BACs 168M02, 209O12, and 113N21 were shotgun-sequenced.

Sequences were analyzed by application of the NIX software tool (www.hgmp.mrc.ac.uk/NIX/). Putative exons and genes were compared at the nucleotide and amino acid levels to known genes by standard GCG programs and by BLAST and FASTA database searches. The relevant part of the contig is deposited under GenBank accession no. AY129241.

Southern Blot Analysis. DNA from individual fish was obtained from pooled organs as described. Five micrograms of genomic DNA was digested with restriction enzymes, separated on 0.8% agarose gels, and blotted onto nylon membranes (Hybond N+, Amersham Pharmacia). Membranes were hybridized either under conditions of moderate stringency [hybridization in 35% formamide, 0.1% Na-pyrophosphate, 50 mM Tris·Cl (pH 7.5), 5 × SSC, 1% SDS, 5 × Denhardt's, 100 μg/ml calf thymus DNA at 42°C, washing in 1 × SSC/1% SDS at 60°C] or high stringency (hybridization in the same buffer except that the formamide concentration was 50% and at the same temperature, washing in 0.1 × SSC/1% SDS at 68°C) with the following probes: *OlaDMRT1*, 4-kb *Eco*RI fragment from cosmid 73K2481; *OlaDMRT1Y,* 1.1-kb *Nhe*I/*Sst*I fragment from phage 2.1 of the subgenomic library; and *HsaDMRT1*, 1.5-kb *Eco*RI fragment from the human *DMRT1* cDNA.

Expression Analysis. Total RNA was extracted from pooled organs of several adult medaka fish or 10–40 pooled total embryos of defined stages (21) by using the TRIZOL reagent (GIBCO/BRL) according to the supplier's recommendation. After DNase treatment reverse transcription was done with 2 or 4 μg total RNA by using Superscript II reverse transcriptase (GIBCO/BRL) and random primers. cDNA from 10 ng (actin) to 200 ng (adult organs) or 600 ng (whole embryos) of total RNA was used for PCR with gene-specific primers: *Ola Actin*, MAct1(TTC AAC AGC CCT GCC ATG TA) and MAct 2 (GCA GCT CAT AGC TCT TCT CCA GGG AG) at an annealing temperature of 60°C for 25 cycles; *Ola DMRT1*, DMT1m (TCC GGC TCC ACA GCG GTC) and DMT1n(CAG ACA GAG GGT TGG GGG G) at an annealing temperature of 64°C for 35 cycles; *Ola DMRT1Y*, DMTYa (GGCCGGGTCCCCGGGTG) and DMTYc (CTG GTA CTG CTG GTA GTT GTG) at an annealing temperature of 64°C for 35 cycles.

RNA from sex-reversed embryos and adults was obtained after treating embryos from day 1 until hatching with 1 mg/ml 17β estradiol in the rearing medium essentially as described (22).

Whole-mount RNA *in situ* hybridization on adult testis was performed according to standard protocols (23). Samples were digested with proteinase K for 2 min before hybridization with a 488-nt *DMRT1Y* antisense riboprobe at 65°C. The riboprobe was generated from a partial cDNA that was obtained by reverse transcription–PCR using the DMTYa and DMTYc primers. Stained tissue samples were paraffin-embedded, sectioned, and counterstained with eosin.

Mapping. A sex-reversed XY female backcross panel was generated as reported (8). A total of 117 backcross progeny were analyzed for four STS markers (Yc-2, Casp6, SL1, Casp3B) that were previously isolated (7, 8, 24) and two phenotypic markers (lf: leucophore, y: male sex). *DMRT1Y* was mapped by PCR using primers DMTk and DMTn (TGA TGC AGC ATT TTG ACA CAT TTA). The products were electrophoresed on 6% acrylamide gels. Segregation of the markers was analyzed with Macintosh MAPMAKER version 2 (25).

Fluorescence *in Situ* Hybridization. BAC clones were labeled separately by standard nick translation using biotin-16-dUTP and digoxigenin-11-dUTP. For two-color hybridization equal amounts of labeled probes were mixed with hybridization solution at a final concentration of 10 μg/ml and used at 100 ng per slide. Before hybridizing with denatured medaka mitotic chromosomes the probe mixture was denatured and preannealed in the presence of excess genomic DNA. Hybridization sites for both probes were simultaneously detected by means of rhodamine-conjugated avidin (Vector Laboratories) and antidigoxigenin (monoclonal)-conjugated fluorescein (Sigma). Chromosomes were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Digitized images of the FITC and rhodamine signals were captured separately and displayed on DAPI-stained chromosomes by using EASY FISH 1.0 software (Applied Spectral Imaging, Mannheim, Germany).

Results

In our studies on sex-determining genes in the medaka we have recently found that the *DMRT1* homologue of medaka is located on an autosome in a gene cluster together with its paralogues, *DMRT 2* and *3* (26). cDNA probes from human and medaka *DMRT1* used on Southern blots of male and female DNA disclosed an additional fragment only in males (Fig. 1 *a–e*). The male-specific fragment was cloned from a partial genomic library and found to be a duplicated version of *DMRT1*, showing 93% identity on the nucleotide and 90% similarity on the amino acid level (Fig. 2). It has a nucleotide identity of 96% (92% amino acids) to the *DMY* gene isolated by Matsuda *et al.* (27). The sequence differences probably are caused by different strains of medaka that were used for analyses. PCR primers specific to the Y-chromosomal copy (designated *DMRT1Y*) were used for a linkage analysis (Fig. 1*f*). *DMRT1Y* was found in all 81 males but not in 57 females of the i-3 strain and in 226 tested females of seven other strains from the Northern and Southern medaka populations. For mapping we used the sex-reversed (XY female × XY male; ref. 8) backcross mapping panel, which spreads the map distances of Y-chromosomal markers in the vicinity of *SD* by a factor of 10 compared with the resolution obtained with a male backcross panel. No recombination between *SD* and *DMRT1Y* was detected (Fig. 1*g*), whereas all earlier described markers map with some distance left and right to *SD* (8, 24). Thus *DMRT1Y* and the male *SD* colocalize on the genetic map (linkage <0.24 cM, equivalent to approximately 125 kb according to refs. 7 and 28).

Sequence analysis of BACs from a genomic library of the HNI strain, cosmids from two independent genomic libraries of Northern (Kaga strain) and Southern (Carbio strain) medaka, and a full-length testis cDNA further confirmed that *DMRT1Y*

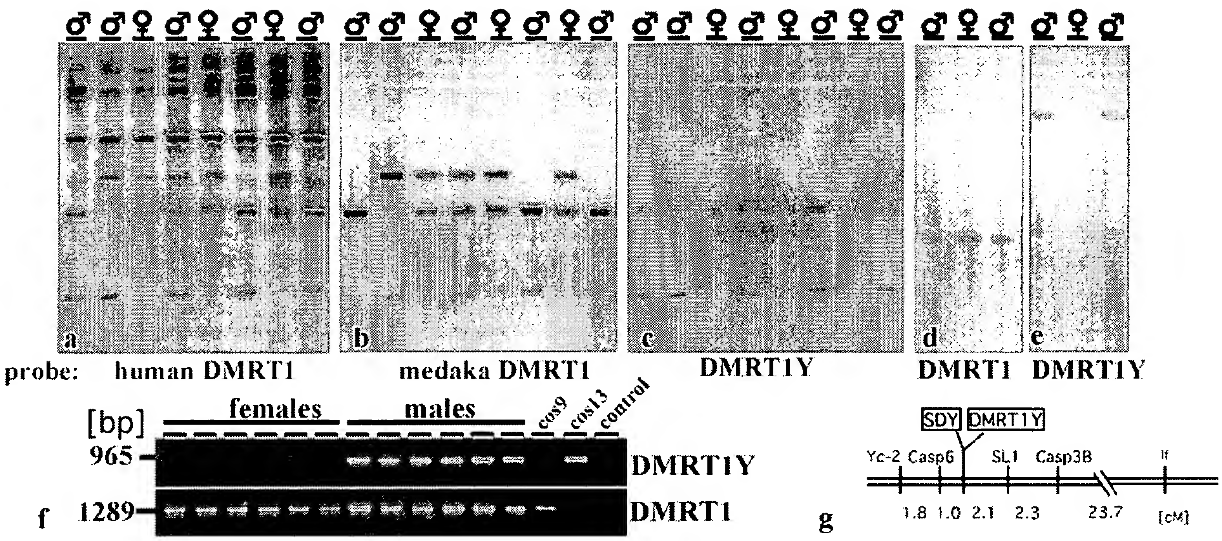

Fig. 1. Southern blot of *Eco*RI-digested male and female DNA with a human *DMRT1* cDNA (*a*), and rehybridized with medaka genomic *DMRT1* (*b*) and *DMRT1Y* (*c*) probes. *DMRT1* and *DMRT1Y* show some cross-hybridization to each other even under conditions of highest stringency because of the high sequence similarity. The hybridization conditions in *a* were moderate stringency and high stringency in *b* and *c*. (*d* and *e*) Hybridization under even further increased stringency conditions of *Pst*I-digested DNAs. (*f*) PCR from DNA of female and male medakas (strain i-3) with *DMRT1Y*- and *DMRT1*-specific primers. Cosmid 9 contains the autosomal *DMRT1* gene (26) and cosmid 13 contains *DMRT1Y*. (*g*) Genetic linkage map of the region flanking the male *SD*, based on meiosis in sex-reversed XY females. Numbers indicate genetic distances in centimorgans (cM). Markers *DMRT1Y* and *SDY* (locus determining the male sex phenotype) showed no recombination.

has all of the features of a functional gene and is not corrupted by mutation. BAC 15H17, which contains *DMRT1Y*, and adjacent BACs were used for fluorescence *in situ* hybridization analysis (Fig. 3) on male and female metaphases. BACs containing the marker sequences flanking the male-determining locus [SL1, Casp3B (data not shown), Casp6 (data not shown)] gave signals on both the X and Y chromosomes. Neighboring BACs that overlap with 15H17 hybridized to the Y and X chromosome as well. However, BAC 15H17 gave a strong hybridization signal only on the Y, but not the X. Weak signals were obtained with BAC 15H17 at the subtelomeric region of a chromosome pair that is equivalent to linkage group 9 of medaka. This is the location of the autosomal *DMRT* cluster.

Analysis of the contiguous sequence of three overlapping BACs (covering 380 kb, roughly equivalent to 0.74 cM; refs. 7 and 28) revealed the size and the borders of the duplicated region on the Y (Fig. 4). Downstream of the Y-specific region a gene is encoded that is the medaka orthologue of human *KIAA0032* (GenBank accession no. BAA04945). This gene (as well as the next one, *KIAA0914*, GenBank accession no. XP_003489) is also present on the X, thus defining the downstream limit of the Y-specific segment. At the upstream border of the Y-specific fragment another copy of *KIAA0032* is present. Thus, the size of the Y-specific fragment is about 260 kb. In the Y-specific region *DMRT1Y* is the only gene that is not corrupted by mutations. There are remnants of three other genes that are also found adjacent to *DMRT1* on the autosome and a fourth one from elsewhere in the genome, but they are all nonfunctional. From the BAC sequences no other functional gene is predicted by using 11 different gene or exon/intron prediction programs. However, a strikingly high number of transposons and other repetitive sequences were noticed, consistent with the expected genetic degeneration and recombinational isolation of the chromosomal region surrounding *SD* on the male-determining chromosome (29).

In adult fish *DMRT1Y* is expressed only in testes like the autosomal *DMRT1* (Fig. 5*a*). The transcript is localized in the Sertoli cells (Fig. 5*b*). During development *DMRT1Y* expression starts at the neurula stage and persists during embryogenesis and larval stages to adulthood (Fig. 5*c*). It is expressed only in male embryos. The expression of the autosomal *DMRT1* starts much later, around day 20.

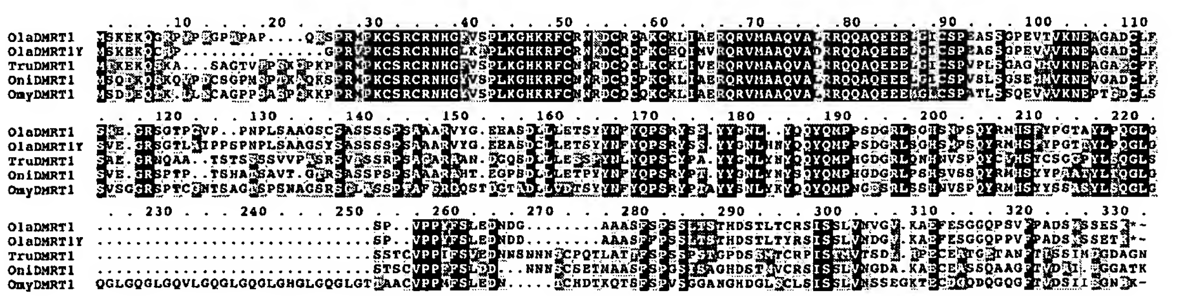

Fig. 2. Sequence comparison of the medaka *DMRT1* and *DMRT1Y* genes with the *DMRT1*s from other teleosts. Ola, *Oryzias latipes* (Medaka); Tru, *Takifugu rubripes*; Oni, *Oreochromis niloticus* (Tilapia); Omy, *Oncorhynchus mykiss* (rainbow trout).

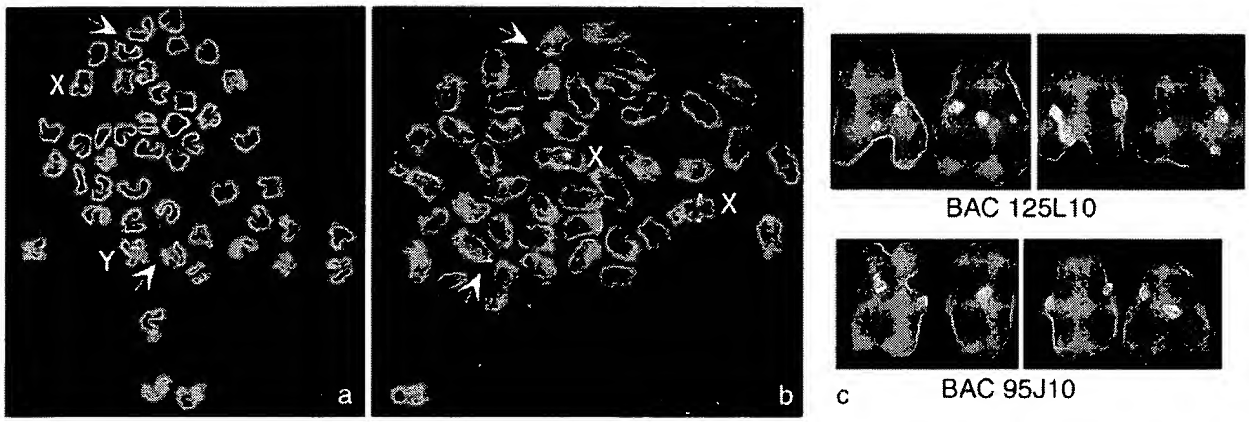

Fig. 3. Identification of the medaka Y chromosome: metaphases from male (*a*) and female (*b*) showing the hybridization signals of two BAC probes (15H17: *DMRT1Y*; 98C17: *SL1*). Note the presence of three hybridization spots for the BAC 15H17 in males as compared with the two spots in female (red signal). The additional fluorescence *in situ* hybridization signal in male is on the Y chromosome. The two relatively weak signals (arrows) in both male and female metaphase spreads represent the autosomal *DMRT1* locus (linkage group 9). The *SL1* marker containing BAC 98C17 detects both sex chromosomes (green signal). (*c*) Two highly enlarged XY chromosome pairs from two metaphases of male medaka showing hybridization to both sex chromosomes of BACs, which contain sequences flanking the Y-specific region on either side (95J10 and 125L10) (red signals). The *SL1* marker containing BAC98C17 (green signals) was used to identify the sex chromosomes.

To analyze whether *DMRT1Y* is an upstream sex-determining gene or a more downstream sex-differentiation gene we analyzed the expression of *DMRT1Y* in XY sex-reversed females. During the estrogen treatment and after hatching the expression of *DMRT1Y* was not affected. The transcript was even detected in the ovary of the adult XY females at levels comparable to testes (Fig. 5*d*).

Discussion

In medaka the pseudoautosomal region of the sex chromosomes is very large. In fact, sex chromosomal crossing-over occurs over the entire length of the chromosome, with the possible exception of the region immediately adjacent to *SD* on the Y chromosome where an extremely high density of markers could not be ordered because of lack of detectable recombination (7, 8, 24). Linkage group 1, which contains *SD*, is the largest one and is equivalent to one pair of homomorphic chromosomes of the largest group. All markers mapped on linkage group 1 (the sex chromosomes) are shared between the X and Y chromosomes, whereas the markers generated from the Y-specific fragment are present only on the Y chromosome (data not shown). This finding indicates that the Y-specific region may be very small. Together with the fact that *DMRT1Y* is highly similar to its ancestor *DMRT1*, which points to a recent rather than to an ancient duplication event, it appears that the sex chromosomes of medaka are at an early stage of evolution.

Gene hierarchy studies in the worm and the fly revealed that the corresponding *DMRT1* homologues *mab-3* and *DSX* are placed at the bottom of the sex-determination cascade. From an evolutionary point of view it appears that the genes at the top of the hierarchy, which rule the mechanism of sex determination, have become involved in this process only relatively recently. However, at least some of the downstream genes, like *DMRT1*, are conserved with respect to sequence and function (2, 30, 31). Based on findings that *DMRT1* is Z-linked in chicken it has been suggested that *DMRT1* in birds has been recruited as an upstream regulatory sex-determining factor (16, 32). In chicken *DMRT1* expression precedes expression of all other potential *SD* genes, is stronger in male than in female gonads, and is evident before the sex differentiation of the gonad anlage starts (18, 19).

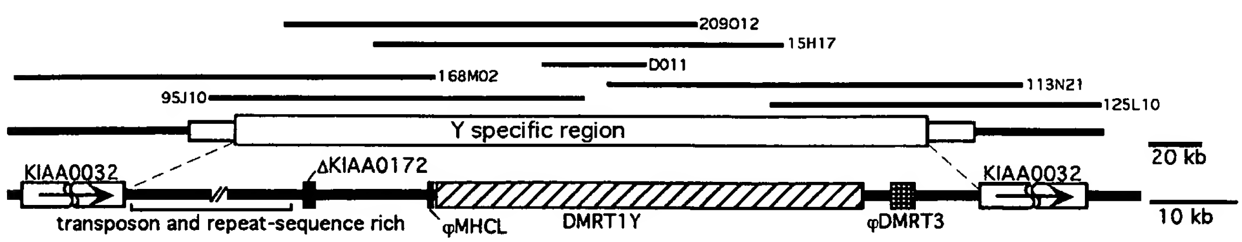

Fig. 4. Schematic representation of the *DMRT1Y*-containing region. Lines above show the analyzed BAC and cosmid clones. Genes and sequences with predicted homology to known genes are shown as boxes: striped, *DMRT1Y*; hatched, *φDMRT3*; light gray, *KIAA0032*; black, *ΔKIAA0172* and *φMHCL*. The region upstream of *ΔKIAA0172* contains only repetitive DNA and sequences with similarity to transposable elements of various organisms. Two genes upstream of *DMRT1*, a myosin heavy chain like gene (*MHCL*) and an ankyrin repeat containing gene (orthologous to human *KIAA0172*), are part of the duplicated fragment from linkage group 9 on the Y. The duplicated Y-chromosomal copy of *MHCL* is, however, destroyed by insertion of a poseidon element, a non-long terminal repeat retroposon (40), in Southern medaka and additionally a *TX-1*-related transposon in Northern medaka. The Y-chromosomal version of *KIAA0172* is corrupted by a deletion that takes out two exons. In addition, the 5′ part of the gene is missing, indicating the border of the duplicated fragment. In intron 4 of *DMRT1Y* an insertion has occurred. This insertion contains a duplicated copy of the putative medaka homologue of the human brain and testes antigen gene *MAP1* that is located on medaka linkage group 19 (M.K., H. Mitani, A.S., and M.S., unpublished work). The Y-chromosomal copy of *MAP1*, however, has a frameshift mutation that leads to a prematurely terminated protein. Downstream of the Y-chromosomal *DMRT1* a copy of *DMRT3* is found. But its coding sequence is lacking the ATG start codon and it has several frameshifts. *DMRT2*, which is the next gene following *DMRT3* on the autosomal cluster, is not found in the Y-specific region.

GENETICS

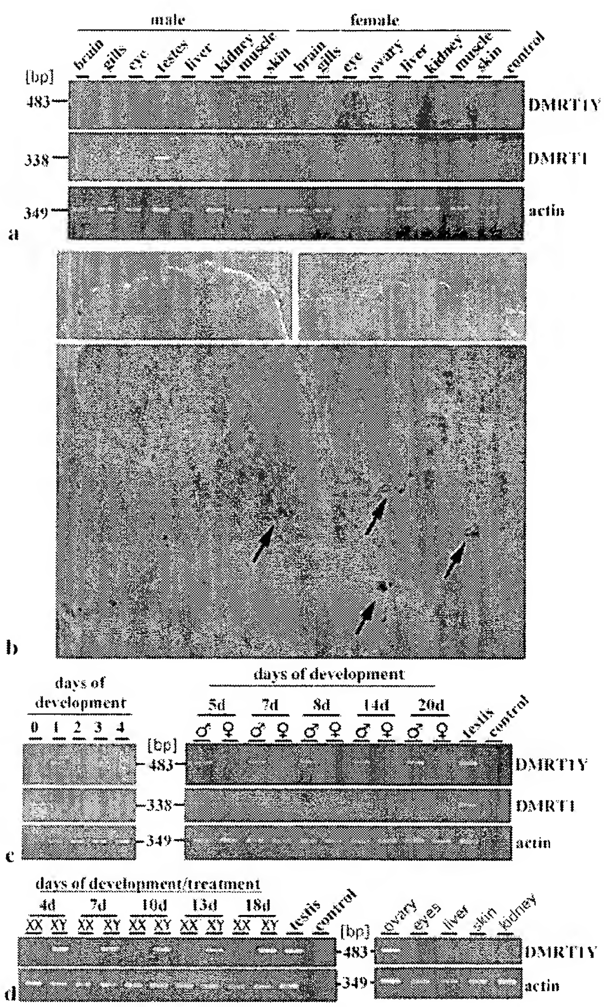

Fig. 5. Expression of *DMRT1Y* and *DMRT1*. (*a*) Reverse transcription–PCR with *DMRT1Y*-specific primers of total RNA from organs of adult male and female medaka. Actin expression was determined for calibration. (*b*) Whole-mount RNA *in situ* hybridization in adult testes with *DMRT1Y* antisense probe (*Upper Left*), *DMRT1* sense control (*Upper Right*), and section of testes showing staining in Sertoli cells (arrows, *Lower*). (*c*) Reverse transcription–PCR of medaka embryos and hatchlings with the same primers as in *a*. (*Left*) Analysis from Carbio strain samples. (*Right*) Analysis from Quart strain samples. (*d*) *DMRT1Y* expression in 17β estradiol-treated Quart embryos and sex-reversed adult XY females.

However, the exact map position of *DMRT1* on chicken Z in relation to the male *SD* locus remains to be determined. In the temperature-dependent sex determination of alligators and turtles, *DMRT1* exhibits the expected expression pattern for a sex-determining gene (19, 33). In rainbow trout it has been shown that *DMRT1* is expressed in the developing male gonad before morphological differentiation (15). In the mouse gonad specific expression of *DMRT1* is detected at embryonic day 9.5, whereas *Sry* expression begins around embryonic day 10.5 (18). Also in humans the simultaneous onset of *DMRT1* and *Sry* expression suggested a role of *DMRT1* in early events of sex determination (34).

What makes *DMRT1Y* in medaka a reasonable candidate for a sex-determination gene? The usual experimental tools to confirm a candidate gene cannot be applied here. In medaka, like in many other fish, sex can be experimentally reversed by steroid treatment or interfering with the activity of sex-differentiation genes. This means that transgenic expression of genes that act downstream in the sex-determination cascade or even sex-differentiation genes will lead to full sex reversal as well. Blocking *DMRT1Y* activity by antisense oligonucleotides cannot be used either, because the gonad is the last organ system to develop in medaka (35), and even morpholinos are not stable enough to be effective (data not shown). Matsuda *et al.* (27) found that a point mutation in the *DMY* (*DMRT1Y*) leads to XY male to female sex reversal. This finding shows convincingly that this gene is necessary for male sexual development. Also in humans the loss of *DMRT1* is connected to XY sex reversals (11–13, 36). *DMRT1* knockout mice revealed that the gene is essential for testis development (37). Thus, the data are not informative whether the gene in medaka has a different (more upstream) or a similar function (more downstream) like that in mammals. Of course, the linkage of *DMRT1Y* to the male *SD* (independently seen in this and another study, ref. 27) is pretty suggestive for its function as the primary sex-determining gene. As genetic mapping has only a certain resolution, a candidate gene may be located very close but not exactly at the locus encoding the phenotype in question. Thus further cumulative evidence should be helpful.

First, our finding of expression of *DMRT1Y* in XY sex-reversed females indicates that *DMRT1Y* is located upstream in the genetic hierarchy and is not one of the male sex-differentiation genes that have to be suppressed by sex-reverting hormone treatment. This experiment, however, does not rule out a difference in activity of *DMRT1Y* in sex-reversed animals, for instance if *DMRT1Y* is regulated posttranscriptionally. Second, the expression pattern of *DMRT1Y* is consistent with a sex-determination function. It is expressed early and exclusively in the male embryo. Most importantly, *DMRT1Y* in medaka is the only functional gene found in the Y chromosome-specific segment at the sex-determining region. The fact that androgen treatment during the sensitive period produces functional XX sex-reversed males that are fully fertile (6) excludes that spermatogenesis or other sex-differentiation genes are present on the Y, unlike the situation in mammals (38, 39). Hence, *DMRT1Y* is not involved in these processes but should have a function as a male sex-determination gene.

Experiments on the biochemical function of *DMRT1Y* are needed to understand how this gene functions in the male sex-determination process. The origin of *DMRT1Y* from an autosomal gene raises the question of what role gene duplication of sex determination and differentiation genes may play in the evolution of sex-determination systems. Such studies in suitable fish models might also help to contribute to our understanding of the function of *DMRTs* in mammals.

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Third Edition

Edited by:

Vernon E Coyne, M Diane James, Sharon J Reid and Edward P Rybicki

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DETECTION OF NUCLEIC ACIDS BY HYBRIDISATION

Contents

INTRODUCTION

Hybridisation is a term used to describe the specific complementary association due to hydrogen bonding, under experimental conditions, of single-stranded nucleic acids. It should more properly be referred to as "annealing", as this is the physical process responsible for the association: two complementary sequences will form hydrogen bonds between their complementary bases (G to C, and A to T or U) and form a stable double-stranded, anti-parallel "hybrid" helical molecule. One may make ones nucleic acid single-stranded for the purpose of annealing - if it is not single-stranded already, like most RNA viruses - by heating it in 0.01M NaCl to a point above the "melting temperature" of the double- or partially-double-stranded form, and then flash-cooling to $\pm$0oC: this ensures the "denatured" or separated strands do not re-anneal.

Alternatively, one may denature DNA reversibly by treatment with 0.5M NaOH: this does not work for

RNA, as this hydrolyses under these conditions.

Why would one want to anneal pieces of nucleic acid?

The answer is simple: nucleic acid hybridisation on membrane filters is a simple, sensitive, and specific means of detecting nucleic acid sequences of interest. One immobilises "target" nucleic acid - denatured so as to be effectively single-stranded - on an absorptive, porous membrane, and then anneals to it an appropriately "tagged" or "labelled" single-stranded probe nucleic acid. After washing off unannealed probe, one detects the immobilised hybrid by means of the label: this is often 32P incorporated into a nucleotide, which allows autoradiographic or scintillometric detection.

One may also use non-radioactive labels and detection systems, for sensitivities of detection down to picogram levels. The system of choice at the moment appears to be the Boehringer Mannheim DIG (digoxigenin) non-radioactive labelling and detection kit, which uses digoxigenin-11-dUTP as a substituted nucleotide which is enzymatically incorporated into DNA.

The mechanism of immobilisation of nucleic acids on membranes is not fully understood: nitrocellulose strongly binds only ss-nucleic acids (ssNA), under conditions of high salt (>1M NaCl), and has to be heated at 80oC in a vacuum to irreversibly attach the NA; nylon membranes (Hybond-N, GeneScreen) bind all nucleic acids under a wide range of salt concentrations, and irreversible or covalent attachment can be achieved by UV irradiation for 5 min or less, or by treatment with 0.4M NaOH.

The complementary association of two strands of polynucleotides

is the basis for replication of all organisms; the complexity inherent in the sequence of the molecules renders the association extremely specific for any molecule longer than sixteen nucleotides. This is easily understood if one considers the combinatorial possibilities of given lengths of "probe" sequence: there is a ¼ chance (4-1) of finding an A, G, C or T (U for RNA) in any given DNA sequence; there is a 1/16 chance (4-2) of finding any dinucleotide sequence (eg. AG); a 1/256 chance of finding a given 4-base sequence. Thus, a sixteen base sequence will statistically be present only once in every

416 bases (=4 294 967 296, or 4 billion):

this is about the size of the human genome, and 1000x greater than the genome size of *E. coli.*

Thus, the association of two nucleic acid molecules - presumed to be at least a few hundred bases long - is an extremely sequence-specific process, far more so than the widely-used specificity of monoclonal antibodies in binding to specific antigenic determinants. The correct annealing of two sequences to each other does, however, depend on the physical and chemical solution conditions under which the reaction takes place.

Melting Temperatures

For example, all double-stranded nucleic acids - whether dsDNA, dsRNA or RNA:DNA hybrids - have specific "melting temperatures", which depend mainly upon their specific guanine+cytosine content, but also upon whether they are DNA, RNA, or a mixture (RNA:RNA hybrids have the highest melting temperatures, followed by DNA:RNA hybrids, then dsDNA), and upon the ionic strength of solution.

The melting temperature is also dependent upon the length of the sequences to be annealed: the shorter the probe sequence, the lower the melting temperature. The degree of sequence mismatch also determines the effective melting temperature of a hybrid: Tm decreases by about 1oC for every 1% of mismatched base pairs. It therefore makes sense to maximise probe length in order to minimise Tm reduction due both to length and degree of sequence mismatch. Under standard conditions of annealing (0.8M NaCl, neutral pH) one may calculate the **melting temperature** ™ of any given DNA hybrid as shown:

Tm = 81.5oC + 0.41(%G + %C) - 550/n

where n=probe length (no. nucleotides).

One can see that the reduction in Tm becomes negligible for probes of length 200 nt or greater. Thus, one may vary the specificity of association of a specific single-stranded "probe" and a target by varying the incubation temperature of the annealing reaction: the higher the temperature, the higher the specificity of the reaction - and the lower the likelihood of annealing taking place.

Hybridisation Stringency

The successful use of nucleic acids as probes for sequences of interest therefore depends upon certain reaction conditions which are in turn determined by the physical properties (ie. length and sequence) of the probe. This leads to the concept of ***stringency*** of hybridisation: one increases the stringency by lessening the likelihood of non-homologous annealing. This can be done by simply increasing the temperature of incubation - bearing in mind that *rate* of hybridisation/annealing is maximal at about Tm - 25oC, and too high a temperature results in very slow annealing. An acceptable compromise is to anneal at a standard temperature (eg. 65oC), and then *wash* the annealed and immobilised hybrid molecules to varying degrees of stringency: the extent to which one should wash can be assessed by repeated autoradiography, if the probe is 32P-labelled, or by repeated colour assay of replicates in the case of non-radioactively labelled probe. Washing stringency may be increased by varying the ionic strength (from 1.0M NaCl to 0.02M), or varying the temperature (ambient to 65oC). One may also include SDS or other detergent in wash and in hybridisation buffers in order to decrease non-specific attachment of probe to the adsorptive membrane. For this reason a *blocking or prehybridisation buffer* is normally used before and during the annealing reaction, to block adsorptive sites on the membrane not occupied by target nucleic acid. This normally consists of buffer salts, detergent, protein, inert polymer material, and DNA.

It is possible to include various other constituents in annealing buffers, designed to increase the hybridisation rate, or the stringency, or both. *Formamide* is a helix destabiliser, and enables one to decrease annealing temperature: the presence of **formamide** decreases the Tm as shown:

TFm = Tm - 0.61(%formamide, w/v)

It is most often used in annealing reactions using RNA as target or probe, and especially with dsRNA hybrids, as these have high Tms which necessitate elevated reaction temperatures. Standard conditions using formamide would be 42oC with 50% formamide content in the annealing buffer. Formamide also decreases the rate of annealing, so one normally includes substances like dextran sulphate - a polyanionic polymer - as "molecular exclusion agents" to decrease the volume of solvent available to the probe. Polyethylene glycol is a far cheaper and equally effective substitute for increasing reaction rate. Too high a concentration of DS or PEG raises "background" or non-specific probe attachment to unacceptably high levels. Their effectiveness is also directly proportional to probe length, and they are useless when oligonucleotides of less than 50 nt in length are used as probes.

Summary

A standard hybridisation reaction, then, consists of ***probing*** an immobilised ***target sequence*** on a membrane with a ***labelled specific probe sequence***: this is done by annealing the probe to the target under (usually) standard "hybridisation conditions" of 0.9M NaCl, 65oC, for 4-16 hr. Probes are usually molecules of DNA or cDNA, a few hundred nt to several kilobases long, cloned into and grown up as recombinant plasmids in *E. coli*, and purified by caesium chloride gradient centrifugation. One may also use nucleic acid directly purified from the organism of interest, but this is only really effective if this is a virus or a plasmid, as otherwise the probe length is too great, and the repeat number is too small to give appreciable signal. In other words, probes should not be too long, as otherwise one needs very high concentrations of nucleic acid in order to guarantee a sufficient number of copies of the sequence in order to give a detectable "signal" for detection purposes.

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Diurnal rhythm of H^+-peptide cotransporter in rat small intestine

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Pan, Xiaoyue, Tomohiro Terada, Megumi Irie, Hideyuki Saito, and Ken-Ichi Inui. Diurnal rhythm of H^+-peptide cotransporter in rat small intestine. *Am J Physiol Gastrointest Liver Physiol* 283: G57–G64, 2002; 10.1152/ajpgi.00545.2001.—In mammals, most physiological, biochemical, and behavioral processes show a circadian rhythm. In the present study, we examined the diurnal rhythm of the H^+-peptide cotransporter (PEPT1), which transports small peptides and peptide-like drugs in the small intestine and kidney, using rats maintained in a 12-h photoperiod with free access to chow. The transport of [^{14}C]glycylsarcosine (Gly-Sar), a typical substrate for PEPT1 by in situ intestinal loop and everted intestine, was greater in the dark phase than the light phase. PEPT1 protein and mRNA levels varied significantly, with a maximum at 2000 and minimum at 800. Similar functional and expressional diurnal variations were observed in the intestinal Na^+-glucose cotransporter (SGLT1). In contrast, renal PEPT1 and SGLT1 showed little diurnal rhythmicity in protein and mRNA expression. These findings indicate that the intestinal PEPT1 undergoes diurnal regulation in its activity and expression, and this could affect the intestinal absorption of dietary protein.

kidney; intestinal absorption; brush-border membranes

DIETARY PROTEIN UNDERGOES a series of degradative steps, resulting in a mixture of free amino acids and small peptides. Numerous studies have shown that absorption of the products of protein digestion in the small intestine occurs primarily in the form of small peptides rather than amino acids (1, 17). Cellular uptake of small peptides (di- and tripeptides) is mediated by H^+-coupled peptide transporter (PEPT1) localized at the brush-border membranes of intestinal epithelial cells (17, 20). In the kidney, two isoforms of peptide transporters (PEPT1 and PEPT2) are expressed and play a significant role in conserving peptide-bound amino nitrogen (9, 20). On the basis of the nutritional importance of peptides, enteral and parenteral solutions of short-chain peptides have been used in clinical settings (13). Thus the clinical relevance of PEPT1 and PEPT2 has received increasing attention in recent years. Furthermore, both peptide transporters can transport several pharmacologically active drugs, such as oral β-lactam antibiotics and the anticancer agent bestatin, and affect their intestinal absorption and therapeutic efficacy (17). All of these findings suggest that the intestinal and renal peptide transporters PEPT1 and PEPT2 play important physiological, pharmacological, and clinical roles.

In mammals, most physiological, biochemical, and behavioral processes vary in a periodic manner with respect to time of day. When amino acids, rather than small peptides, were considered to be important as nutritional elements for protein homeostasis, there were a number of reports on the circadian rhythm of amino acid metabolism, such as plasma levels of amino acids (11), amino acid transport and metabolism in the liver (4, 45), and intestinal absorption of L-histidine (12). These studies demonstrated that the highest values or activities were observed in dark phases, and these diurnal changes might be assumed to occur in nocturnal animals feeding mainly at night. However, there have been few reports on the diurnal rhythms for transport and metabolism of small peptides, although the physiological and clinical significance of small peptides has been recognized.

In the present study, we focused on the diurnal rhythms of intestinal absorption of small peptides and its molecular mechanism. To achieve this, diurnal changes of [^{14}C]glycylsarcosine (Gly-Sar) transport in the small intestine were examined. Expressional changes of PEPT1 mRNA and protein in the small intestine were also investigated. Furthermore, we compared the expressional changes of intestinal PEPT1 to those of renal PEPT1, to examine the tissue specificity of circadian rhythms. Diurnal rhythms of the Na^+-glucose cotransporter (SGLT1) in the small intestine and kidney were also examined to compare with those of PEPT1, because the intestinal SGLT1 was reported to show a circadian rhythm (30, 42).

MATERIALS AND METHODS

Animals. Animal experiments were performed in accordance with the Guidelines for Animal Experiments of Kyoto

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University. Male Wistar rats (160–180 g) were housed in an air-conditioned room at 22 ± 0.5°C with a 12-h lighting schedule (800–2000). Animals were kept for at least 1 wk before the initiation of any experiments and were allowed free access to water and standard laboratory chow.

Materials. [^{14}C]Gly-Sar (1.78 GBq/mmol) was obtained from Daiichi Pure Chemicals (Ibaraki, Japan). [^{14}C]methyl-α-D-[U-^{14}C]glucopyranoside (αMG); (9.66 GBq/mmol) was supplied by Moravek Biochemicals (Brea, CA). Gly-Sar was purchased from Sigma (St. Louis, MO). All other chemicals used were of the highest purity available.

In situ loop technique. We examined [^{14}C]Gly-Sar transport by the in situ loop technique at 1200 and 2400. A cannula with a polyethylene tube was inserted in the portal artery. A duodenum loop 10 cm in length was prepared, and then [^{14}C]Gly-Sar (40 nmol·ml^{-1}·kg body wt^{-1}) was introduced into the loop with a microsyringe. Blood was withdrawn from the portal artery at designated times. Blood samples were centrifuged for 2 min at 14,000 *g*, and 50 μl of plasma was solubilized in 0.5 ml of NCS II (Amersham Pharmacia Biotech, Uppsala, Sweden). Radioactivity was determined in 5 ml of ACS II (Amersham Pharmacia Biotech) by liquid scintillation counting.

Preparation of intestinal segments and uptake experiments. Rats were killed at different times during a 24-h period (400, 800, 1200, 1600, 2000, and 2400), and the intestinal segments for the uptake experiments were quickly prepared according to a previous report (24) with some modifications. Isolated duodenum was everted, divided into small segments 5–10 mm in length, and fixed over polyethylene tubes with an outer diameter of 4 mm. Everted intestinal segments were preincubated with incubation medium under an atmosphere of 100% oxygen. The composition of the incubation medium was as follows (in mM): 129 NaCl, 5.1 KCl, 1.4 $CaCl_2$, 1.3 NaH_2PO_4, and 1.3 Na_2HPO_4 (pH 6.0). After preincubation, each intestinal segment was placed in 1 ml of incubation medium containing [^{14}C]Gly-Sar (20 μM) or αMG (100 μM). The uptake experiments were carried out at 37°C an atmosphere of 100% oxygen. After incubation for 3 min, each segment was rapidly washed with ice-cold incubation medium, blotted on filter paper, weighed, and solubilized in 0.5 ml of NCS II. Radioactivity was then determined in 5 ml of ACS II by liquid scintillation counting.

Antibodies and Western blot analysis. Rabbit anti-PEPT1 antibody was raised against the 15 COOH-terminal amino acids of rat PEPT1 (31). Rabbit anti-PEPT2 antibody was raised against synthetic peptides corresponding to amino acids 697–710 of rat PEPT2 (39). Rabbit anti-SGLT1 antibody (a gift of M. Kasahara) was raised against synthetic peptides corresponding to amino acids 564–575 of rabbit intestinal SGLT1 (40). Goat antivillin polyclonal IgG was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). While the animals were under anesthesia, the duodenum and kidney were removed at specified times. The duodenum was flushed with cold PBS, and the mucosa was scraped. The kidney was decapsulated, and slices of renal cortex were prepared with a Stadie-Riggs microtome. A portion of the mucosa and renal slices were rapidly frozen in liquid nitrogen for later preparation of brush-border membranes and total RNA. Brush-border membranes from rat small intestine and kidney cortex were prepared as described previously (16, 29). The membrane fractions were separated by SDS-PAGE and analyzed by Western blotting with each antibody as reported (23, 26, 31, 39). Relative amounts of band in each reaction were determined densitometrically using Image 1.61 (National Institutes of Health, Bethesda, MD).

Immunohistochemistry. Immunohistochemistry was performed as described previously (26, 27). Briefly, fresh specimens were cut transversely, and the lumens were washed with PBS. Samples were embedded in optimal cutting temperature compound (Sakura Finetechnical, Tokyo, Japan), rapidly frozen in liquid nitrogen, and stored at −80°C until used. Frozen sections were cut with a cryostat (5-μm thick), mounted on glass slides, fixed in ethanol for 15 min at −15°C, and washed with PBS at room temperature. The sections were then covered with 5% normal goat serum for 10 min and incubated with anti-PEPT1 antibody at a dilution of 1:250 for 1 h. After being washed with PBS, the sections were next incubated with 3 μg/ml of Cy3-labeled donkey anti-rabbit IgG (CALTAG Laboratory, San Francisco, CA) for 1 h and then washed with PBS. These sections were examined with a BX-50-FLA fluorescence microscope (Olympus, Tokyo, Japan) at a magnification of ×100. Images were captured with a DP-50 charge-coupled device (CCD) camera (Olympus) using Studio Lite software (Olympus). After the fluorescence images had been captured, they were processed and analyzed with IP Lab Spectrum image analysis software (Signal Analytics, Vienna, VA).

Northern blot analysis. Total RNA was extracted using the guanidine isothiocyanate method (7). Each amount of total RNA was electrophoresed in 1% denaturing agarose gel containing formaldehyde and transferred onto nylon membranes. The quality of the RNA was assessed by ethidium bromide staining. After transfer, blots were hybridized at high stringency [50% formamide, 5× sodium chloride-sodium phosphate-EDTA (SSPE) (1× SSPE is 0.15 M NaCl, 10 mM NaH_2PO4, and 1 mM EDTA), 5× Denhardt's solution, 0.2% SDS, and 10 μg/ml herring sperm DNA at 42°C] with PEPT1, PEPT2, SGLT1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA fragments labeled with [α-^{32}P]dCTP as probes. Each probe was already prepared, sequenced, and used for Northern blot analysis (31, 32, 41). After being hybridized, the blots were washed several times in 2× SSC (1× SSC is 0.15 M NaCl, and 15 mM sodium citrate, pH 7.0)/0.1% SDS at room temperature. Dried membranes were exposed to the imaging plates of a Fujix Bio-Imaging Analyzer BAS 2000 II (Fuji Photo Film).

Data analysis. Data were analyzed statistically by the nonpaired *t*-test or one-way ANOVA followed by Fisher's test when multiple comparisons were needed.

RESULTS

[^{14}C]Gly-Sar absorption by in situ intestinal loops at 1200 and 2400. First, we examined the intestinal absorption of [^{14}C]Gly-Sar by in situ intestinal loops in the middle of the light phase (1200) and the middle of the dark phase (2400). Figure 1 shows the mean portal vein concentrations after intraduodenal administration of [^{14}C]Gly-Sar. The initial absorption rate of [^{14}C]Gly-Sar was significantly faster at 2400 than at 1200.

Uptake studies by rat intestinal segments. We then examined the uptake of [^{14}C]Gly-Sar by intestinal segments at different times during a 24-h period. αMG was also measured. Accumulation of both substrates into intestinal segments increased linearly with time up to at least 3 min (data not shown). To evaluate the transporter-mediated specific uptake, nonspecific uptake was evaluated by measuring in the presence of inhibitors (Fig. 2, *A* and *B*). As shown in Fig. 2*C*, the

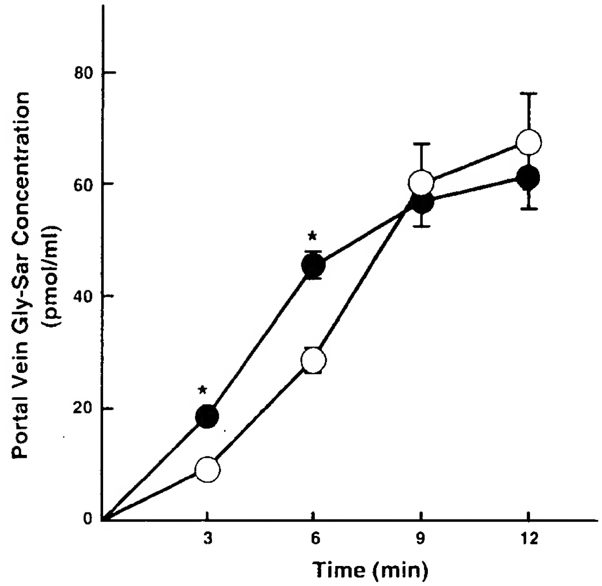

Fig. 1. Time course of portal vein [^{14}C]glycylsarcosine ([^{14}C]Gly-Sar) concentration at 1200 (○) and 2400 (●) in the in situ duodenum loop experiment. Each point represents the mean ± SE of 10 rats, *P < 0.05, significantly different from 1200.

specific uptake of [^{14}C]Gly-Sar tended to be lower in the light phase than the dark phase. For example, the [^{14}C]Gly-Sar specific uptake value was significantly greater at 2400 h (6.1 ± 0.6 nmol/g tissue) than at 1200 h (5.0 ± 0.5 nmol/g tissue) (n = 15 segments from 5 rats; P < 0.05). A similar result was obtained in the [^{14}C]αMG uptake experiments (Fig. 2D).

Diurnal variation of PEPT1 and SGLT1 proteins in the duodenum. To determine whether the rhythmicity of [^{14}C]Gly-Sar uptake was linked to expressional changes of PEPT1 protein, we performed Western blot analysis using intestinal brush-border membranes. As shown in Fig. 3A, the intestinal PEPT1 protein level was highest at 2000 and lowest at 800. A similar pattern of expression was observed for SGLT1 protein (Fig. 3B), and this result was consistent with the previous reports (30, 42). In contrast to both proteins, villin, which is a cytoskeletal marker protein, did not change in expressional level throughout the day (Fig. 3C).

We then analyzed the rat PEPT1 protein expression at different times by immunofluorescence microscopy using a cooled-CCD camera. Immunofluorescence images were captured under the completely same conditions, as previously reported (27). As shown in Fig. 4, localization of PEPT1 is confined to the brush-border membranes of absorptive epithelial cells, and labeling intensity was much stronger at 1600–2400 than at other times.

Diurnal variation of PEPT1, PEPT2, and SGLT1 proteins in the kidney. The diurnal variation in the expression of PEPT1 and SGLT1 protein in the kidney was tested further (Fig. 5, A and C). In contrast to their expressional rhythmicity in the duodenum, levels of PEPT1 and SGLT1 protein expression in the kidney did not vary appreciably during the 24-h period. The protein level of PEPT2, which is another H$^+$-peptide transporter predominantly expressed in the kidney, also showed little diurnal variation (Fig. 5B).

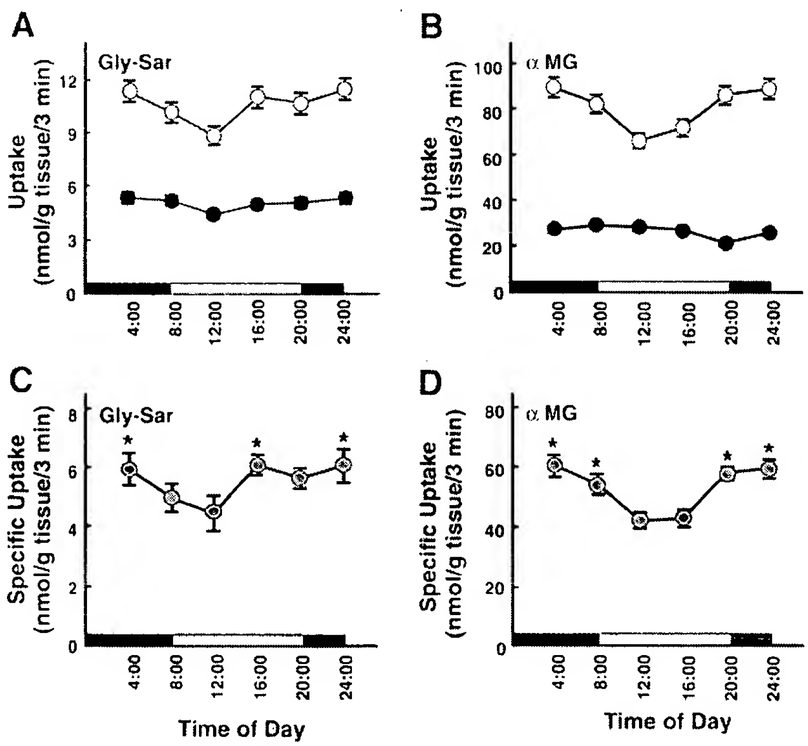

Fig. 2. Uptake of [^{14}C]Gly-Sar (A), and [^{14}C]methyl-α-D-[U-^{14}C]glucopyranoside (αMG) (B) by everted duodenum of rats during a 24-h period (400, 800, 1200, 1600, 2000, and 2400). Each segment was incubated with [^{14}C]Gly-Sar (20 μM) and [^{14}C]αMG (100 μM) in the absence (○) or presence (●) of 20 mM Gly-Sar (A) and glucose (B) for 3 min at 37°C. C (Gly-Sar) and D (αMG) show the specific uptake of each substrate. Specific uptake was calculated by subtracting the nonspecific uptake (●) from the total uptake (○). Open bars and closed bars show light and dark phases, respectively. Each point represents the mean ± SE of 15 measurements from 5 separate experiments. *P < 0.05, significantly different from the lowest value (1200).

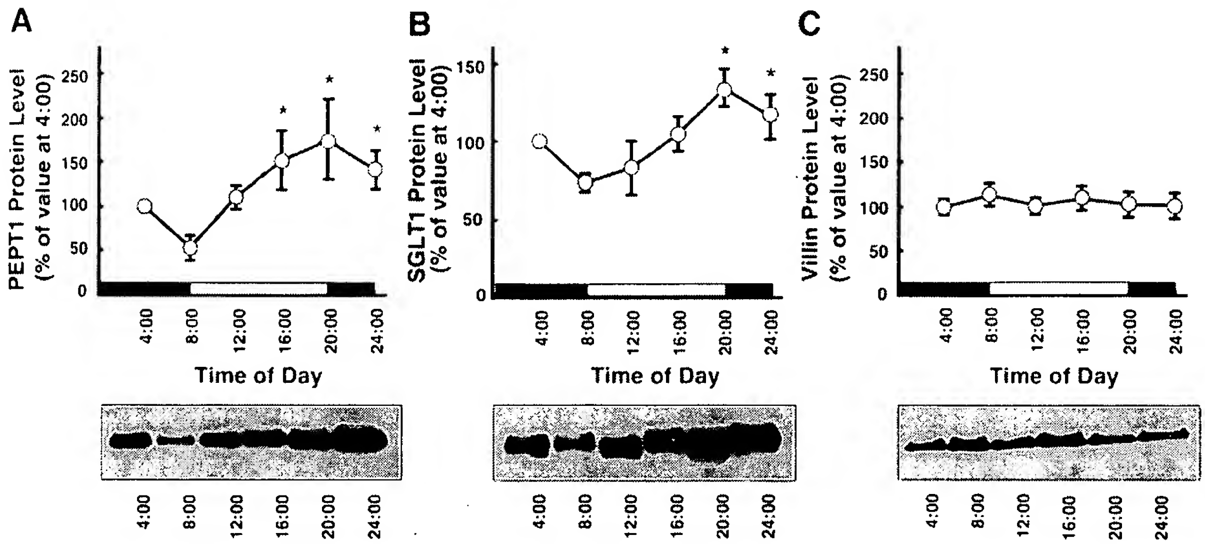

Fig. 3. Diurnal variation of PEPT1 (*A*), SGLT1 (*B*), and villin (*C*) protein expression in rat duodenum. Rats were killed at the indicated time, and brush-border membranes of the rat duodenum were prepared. Membrane proteins (5 μg/lane) were probed with each antibody as described in MATERIALS AND METHODS. Film intensity signals were subjected to scanning densitometry, and protein abundances were expressed as a percentage of the value at 400. Open bars and closed bars show light and dark phases, respectively. Each point represents the mean ± SE of 5 preparations. *$P < 0.05$, significantly different from the lowest value (800).

Diurnal variation of PEPT1 and SGLT1 mRNAs in the duodenum. To assess whether diurnal variation in PEPT1 expression occurred at the transcriptional level, Northern blot analysis was carried out. As shown in Fig. 6*A*, significantly more PEPT1 mRNA was present in the small intestine from 1600 to 2400 than at other times. Similar expressional changes were observed for the SGLT1 mRNA (Fig. 6*B*). On the other hand, the GAPDH mRNA level exhibited no significant diurnal variations (Fig. 6*C*).

Diurnal variation of PEPT1, PEPT2, and SGLT1 mRNA in the kidney. We further investigated the diurnal rhythm of PEPT1, PEPT2, and SGLT1 mRNA expression in rat kidney. As shown in Fig. 7, mRNAs of all the transporters exhibited no apparent diurnal variation.

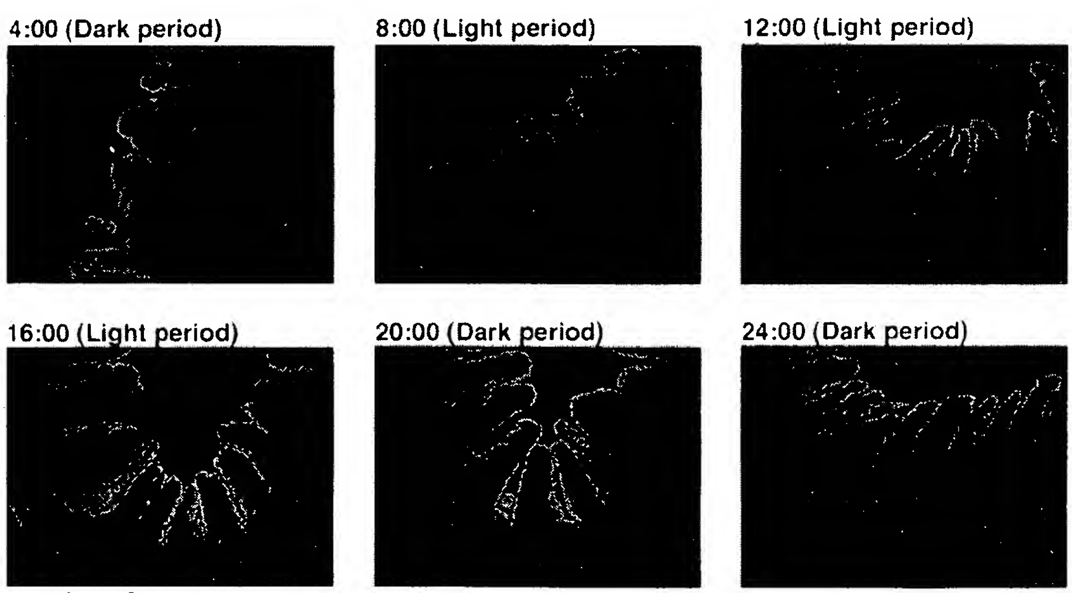

Fig. 4. Immunohistochemical analysis of the diurnal variation of PEPT1 protein expression in rat duodenum. Tissue sections obtained from rats killed at the indicated times were processed as described in MATERIALS AND METHODS. Pictures were taken under the same conditions to allow comparison of labeling intensities. Typical pictures from 3 separate experiments are shown.

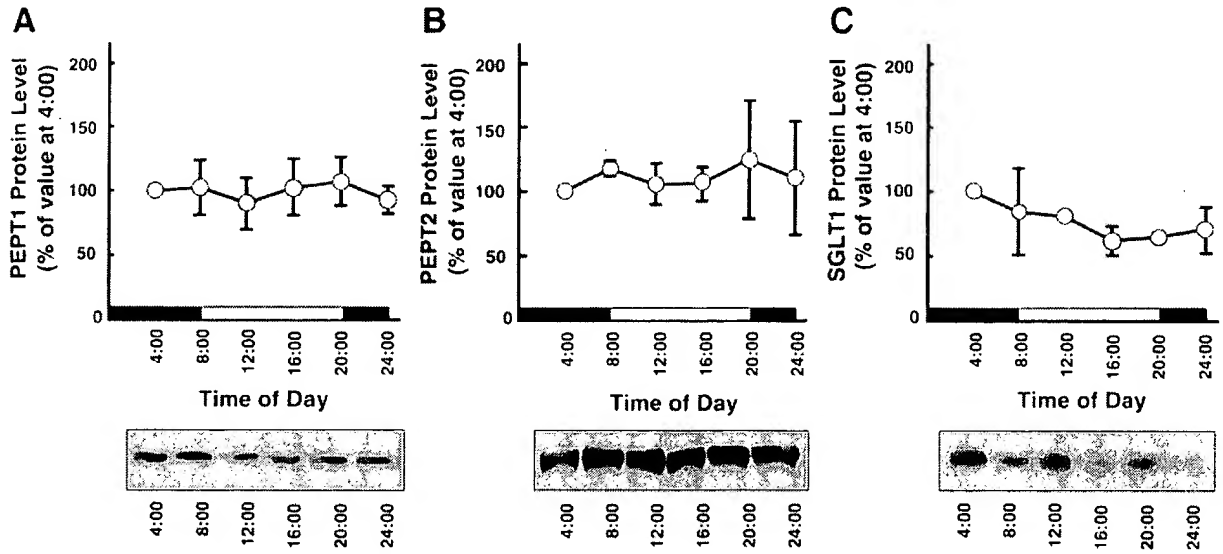

Fig. 5. Diurnal variation of PEPT1 (*A*), PEPT2 (*B*), and SGLT1 (*C*) protein expression in rat kidney cortex. Rats were killed at the indicated times, and brush-border membranes of the rat kidney cortex were prepared. Membrane proteins (30 μg/lane) were probed with each antibody as described in MATERIALS AND METHODS. Film intensity signals were subjected to scanning densitometry, and protein abundances were expressed as a percentage of the value at 400. Open bars and closed bars show light and dark phases, respectively. Each point represents the mean ± SE of 5 preparations.

DISCUSSION

Intestinal PEPT1 is physiologically regulated by various factors including dietary conditions (10, 15, 27, 34), hormones (6, 43), growth factors (25), and development (33). Dietary regulation of intestinal PEPT1 has been extensively investigated (10, 15, 27, 34). For example, we previously demonstrated that short-term starvation markedly increased the amount of PEPT1 protein, whereas dietary administration of amino acids reduced the amount (27). Taking these findings into consideration, it is expected that food content and feeding schedule affect the diurnal rhythmicity of intestinal PEPT1. The aim of the present study is to clarify whether rat PEPT1 shows a diurnal rhythmicity under standard environmental conditions. Therefore, we performed each experiment using rats main-

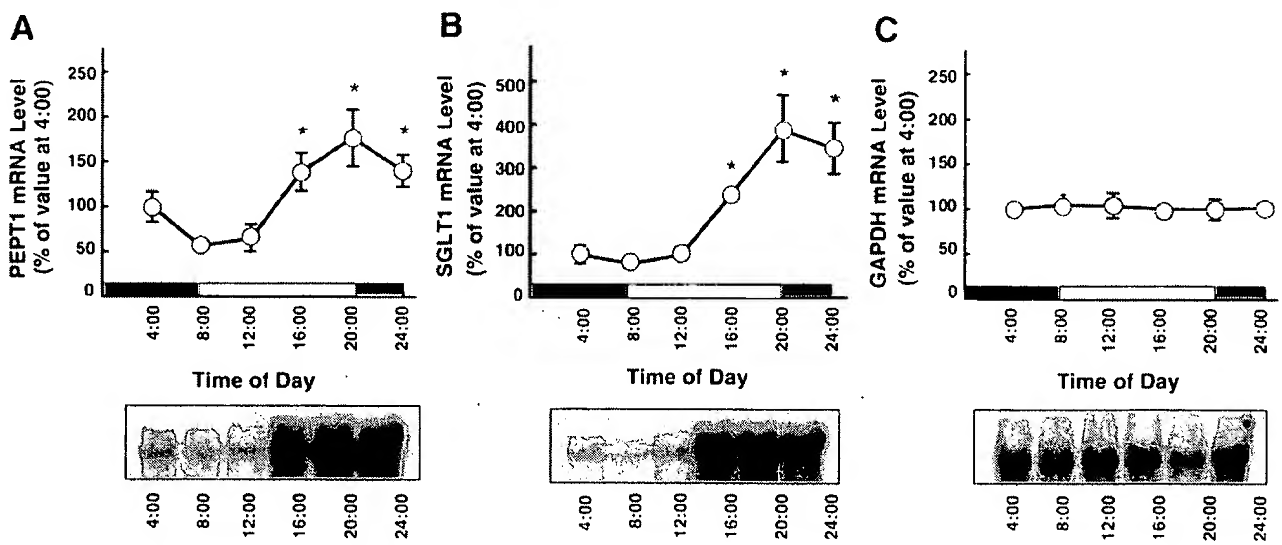

Fig. 6. Diurnal variation of PEPT1 (*A*), SGLT1 (*B*), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (*C*) mRNA expression in rat duodenum. Northern blot analyses with RNA extracted from the duodenum of rats killed at the times indicated were performed as described in MATERIALS AND METHODS. PEPT1 and SGLT1 message levels were quantified by scanning densitometry, corrected for loading using GAPDH, and expressed as a percentage of the value at 400. Open bars and closed bars show light and dark phases, respectively. Each point represents the mean ± SE of 5 preparations. *$P < 0.05$, significantly different from the lowest value (800).

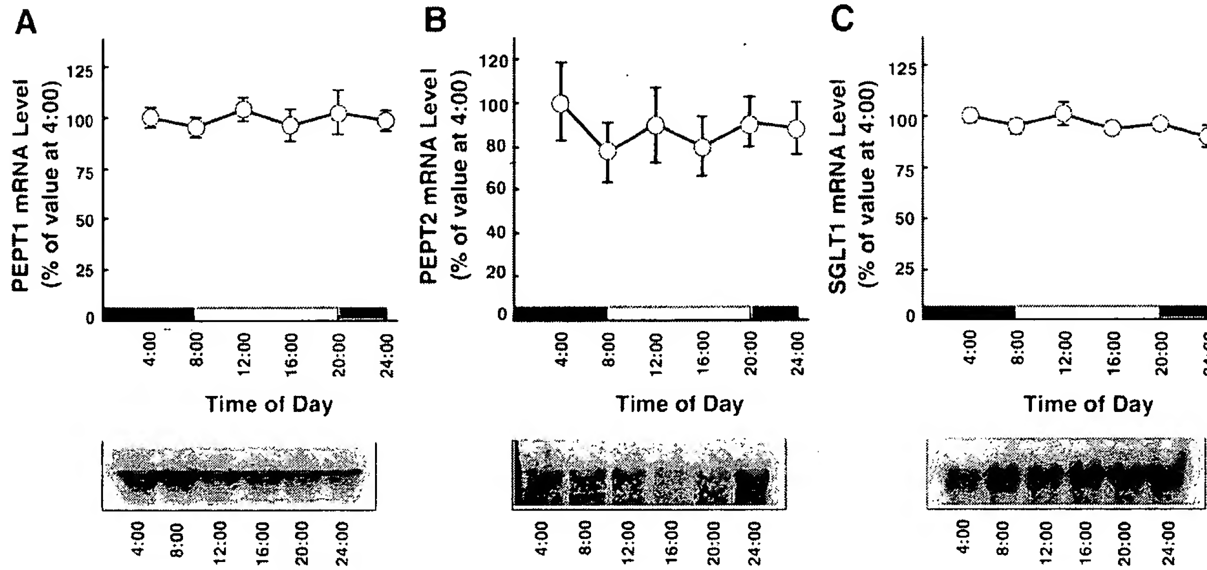

Fig. 7. Diurnal variation of PEPT1 (*A*), PEPT2 (*B*), and SGLT1 (*C*) mRNA expression in rat kidney cortex. Northern blot analyses and quantification of signals were carried out as described in Fig. 6. Open bars and closed bars show light and dark phases, respectively. Each point represents the mean ± SE of 5 preparations.

tained in a 12-h photoperiod with free access to water and standard laboratory chow.

The present study has demonstrated that transport activity and expression level (both mRNA and protein) of intestinal PEPT1 and SGLT1 showed diurnal rhythm. Transport activity and expression levels of both transporters appeared to be higher in the dark phase than the light phase. A similar diurnal rhythm has been observed for various intestinal digestive and absorptive activities, such as sucrase-isomaltase (37), lactase (38), γ-glutamyltransferase (38), alkaline phospatase (38), and glucose and L-histidine transport (12). In addition to the above functional rhythmicity, recent studies have clarified that the expressional rhythms of SGLT1 and facilitative glucose transporters (GLUT2 and GLUT5) are related to these functional rhythms (8, 30, 42). Because rodents show a nocturnal feeding behavior, all of these diurnal rhythms, including the PEPT1 rhythmicity, could seem reasonable for the preparation of nocturnal dietary load.

In the present study, we found that the diurnal rhythm of the expression of intestinal PEPT1 protein is linked with the periodicity of the transcription of the PEPT1 mRNA. But it remains unclear what factors regulate the transcription of intestinal PEPT1. In the case of SGLT1, Tavakkolizadeh et al. (42) suggested two distinct and separate pathways regulating the expression and function in intestinal epithelial cells. One pathway is the utilization of gut luminal signals (presumably food intake) to induce the diurnal variation. This mechanism may be involved in the regulation of PEPT1 rhythmicity, because the dietary regulation of intestinal PEPT1 was reported (10, 15, 27, 34). The second is a daily anticipatory mechanism preparing the intestine for an expected increase in nutrients before exposure to the luminal contents. This mechanism may also contribute to the diurnal rhythm of intestinal PEPT1, because the PEPT1 mRNA level in the small intestine has begun to increase at 1600 before the onset of feeding. It was reported that the bulk of food ingestion occurs in the first 4–6 h of the dark phase when rodents are fed ad libitum (44). It is, therefore, hypothesized that these two factors have complexly influenced the diurnal variation of the intestinal PEPT1 expression.

Rhoads et al. (30) demonstrated that the periodicity in the activity of hepatocyte nuclear factor 1 (HNF-1) contributed to the circadian rhythm of SGLT1 transcription. Because it was reported that there is a potential site for HNF-1 in the rat PEPT1 promoter region (34), this factor may be involved in the daily anticipatory mechanism of intestinal PEPT1 expression. A neuroendocrine mechanism, such as insulin circadian variation, was also proposed to affect the glucose absorption in rats (5, 14). Likewise, insulin was demonstrated to regulate the PEPT1 function by increasing the population of PEPT1 protein in membranes (43). These findings suggest that insulin circadian variation may affect the PEPT1 expression and function, although this mechanism is not involved in the transcriptional regulation. More recently, Buyse et al. (6) reported that PEPT1-mediated epithelial transport of dipeptides and cephalexin is enhanced by gastric leptin, the *ob* gene product, on the luminal side of the small intestine. Although the diurnal rhythm of gastric leptin secretion into the lumen has not been investigated, there are various reports on the circadian rhythm of the plasma leptin levels (2, 3, 22). Thus the luminal leptin secreted by the stomach could be involved in the diurnal rhythm of intestinal PEPT1 expression.

In contrast to the intestinal PEPT1 and SGLT1 expression, the mRNA expression of both transporters in the kidney showed little diurnal rhythmicity. Crypt-villus turnover in the intestine has been demonstrated to show a circadian rhythm; i.e., enterocyte differentiation is increased and peaks at 300 in a 12:12-h light-dark cycle beginning at 600 (35). In addition, the length of the villus and the number of mature enterocytes peak before the onset of feeding (36). However, to our knowledge, there are few reports on the circadian rhythms of cell differentiation and number in renal tubular cells. It is, therefore, assumed that some distinct features of cell dynamics between the intestinal and renal epithelial cells contribute to the different diurnal rhythmicities of the intestinal and renal PEPT1 and SGLT1 expressions. Alternatively, transcription factors, such as HNF-1 may have different effects in the small intestine than in the kidney. Clarification of the regulatory mechanisms of PEPT1 and SGLT1 expression in both tissues should be useful for the understanding of the circadian rhythm of PEPT1 and SGLT1 expression in the small intestine.

Several drugs vary in potency and/or toxicity on the basis of the rhythmicity of biochemical, physiological, and behavioral processes (19, 21). For example, chronopharmacotherapy with interferon-α in mice has been studied, and the dosing time-dependent change in antiviral activity was partly explained by the diurnal variation of pharmacokinetic parameters of interferon-α (18, 28). Because intestinal PEPT1 mediated a wide variety of peptide-like drugs, such as oral β-lactam anitibiotics and the antivirus agent valacyclovir (17), the absorption rate and therapeutic efficacy of these drugs would depend largely on the activity of PEPT1. Although further study is needed, there is a possibility that the pharmacokinetic profiles and therapeutic efficacy of these drugs may be affected by the dosing time schedule.

In conclusion, the present study has demonstrated that the functional activity and expression of intestinal, but not renal, PEPT1 show a diurnal rhythm as well as SGLT1. The diurnal rhythm of intestinal PEPT1 expression occurred in the transcription.

We thank Dr. K. Takata, Department of Anatomy and Cell Biology, Gunma University School of Medicine, for helpful discussion about the immunohistochemistry of PEPT1 in the duodenum.

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First Named Inventor	:	Michael E. Spurlock	
Appln. No.	:	09/928,522	
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Docket No.	:	LL31.12-0015	

EXHIBIT N

of

DECLARATION

submitted under 37 C.F.R. 1.132

PowerPoint Presentation entitled Northern and Southern Blotting by Todd Lamitina, Research Fellow in the Department of Anesthesiology, Research Division, at Vanderbilt University Medical Center (Obtained on December 1, 2004 from the Internet at: http://bret.mc.vanderbilt.edu/igp/html/Methods_2003/Blotting.ppt)

Northern and Southern blotting

- Understanding the terminology
- 6 steps of a blot experiment
  - Sample preparation and gel
  - Transfer
  - Blot fixation
  - Making a probe
  - Probe hybridization
  - Blot visualization
- Experimental interpretation – the pros and cons of blots
- Variations on a theme – other methods that utilize the blotting strategy

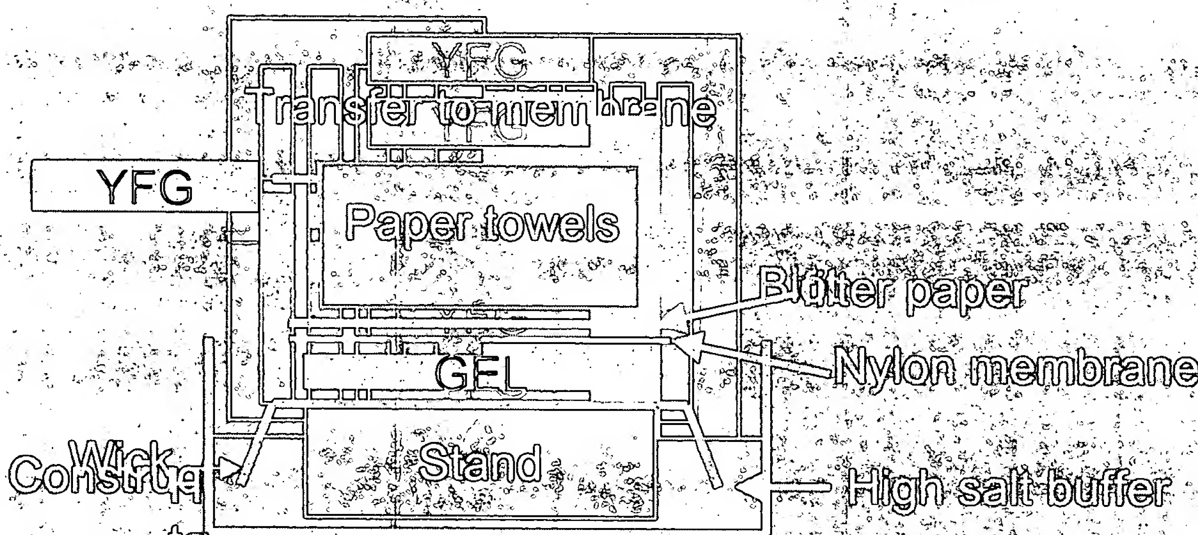
Southern blots

- Named after Brit biochemist Edwin Southern (alive and publishing!)
  - Southern EM. Detection of specific sequences among DNA fragments separated by gel electrophoresis.
    J. Mol Biol. 1975 Nov 5;98(3):503-17.
- A technique for identifying specific sequences of DNA in which DNA fragments are separated by electrophoresis, transferred to a membrane, and identified with a suitable probe.
- Detects restriction fragments following a restriction enzyme digest of genomic DNA (RFLPs)

Northern blots

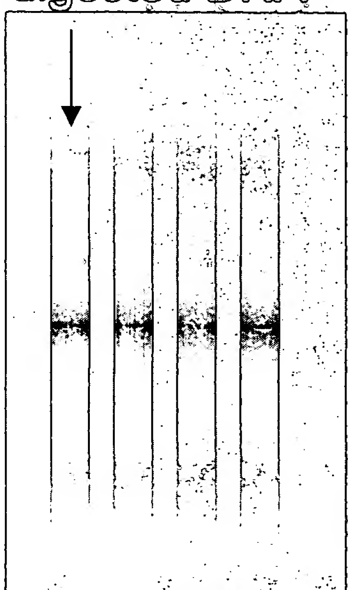
- Name is derived from a LAME scientific sense of humor – a word play on the term "Southern" blot. The term "Western" blot also stems from the same LAME sense of humor
- A technique for identifying specific sequences of RNA (usually mRNA) in which RNA molecules are separated by electrophoresis, transferred to a membrane, and identified with a suitable probe (RNA or cDNA)
- Detects mRNA molecules

Basic proceedure for a Northern or Southern blot



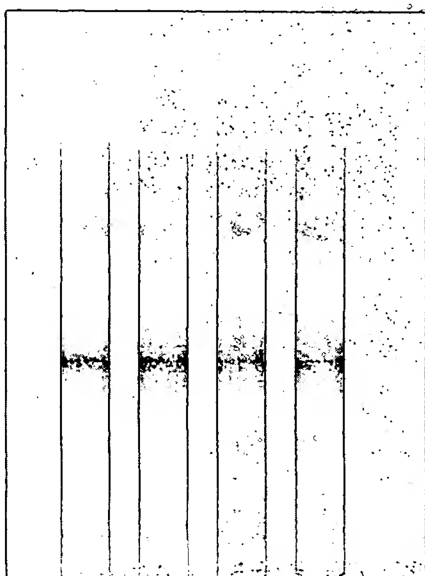
Proper transfer setup

Sample preparation - Southern



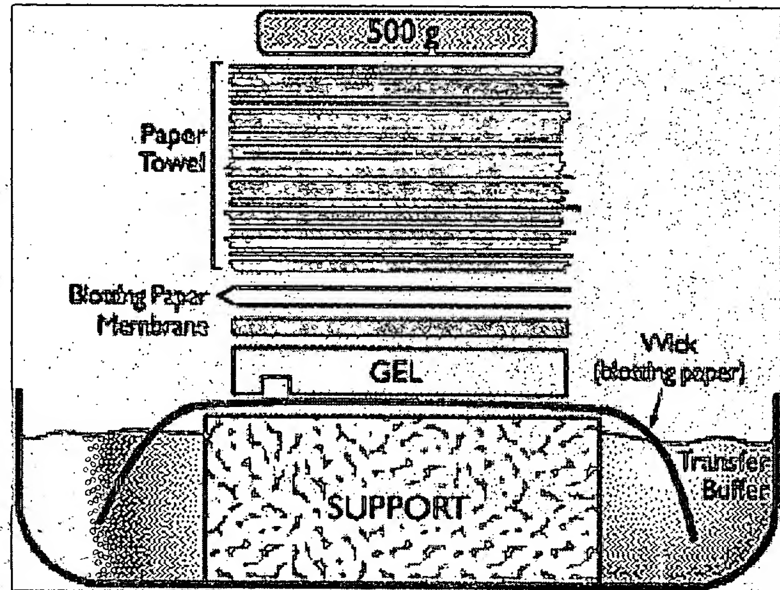
- DNA is HUGE – way too big to enter an agarose gel
- Use a restriction enzyme to break it into smaller pieces (also generates sequence specific patterns – mutations alter these patterns)
- Both gel and buffer are is 1X TAE or 5X TBE

Sample preparation - Northern


Formaldehyde gel

- RNA can be difficult to work with. Always wear gloves and use RNAase free solutions (DEPC-treated water) and glassware (oven baked for at least 2 hours at 180°)
- AUTOCLAVING DOES NOT REMOVE RNAase!!!!!!
- Use polyA enriched mRNA for maximum sensitivity
- Formamide and formaldehyde in the loading buffer and gel prevent formation of RNA secondary structure
- Gel buffer uses 1X MOPS
- For both Northerns and Southerns, at least 1 lane should contain molecular weight markers

Setting up a transfer



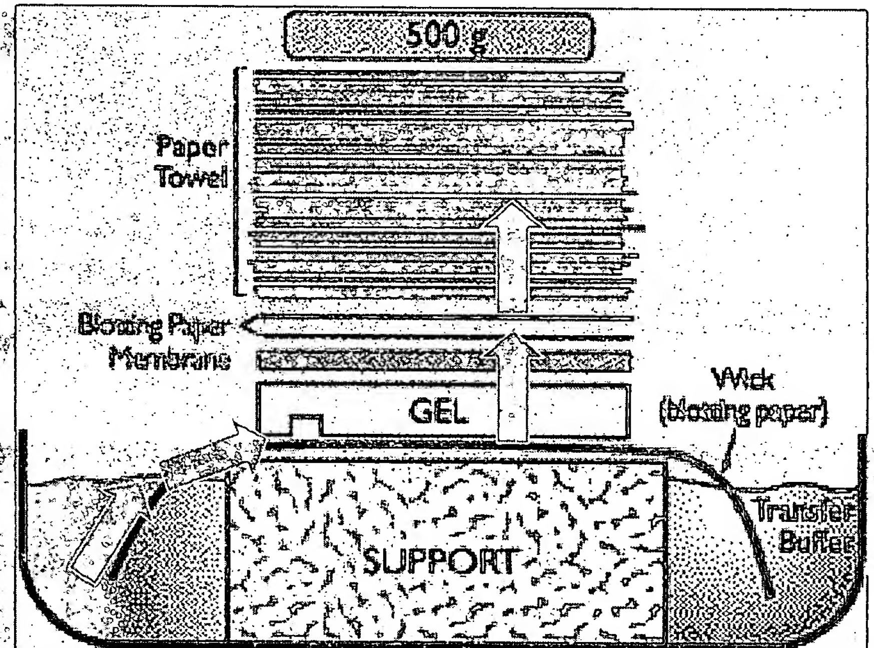
Important points

Preparing the gel for transfer
Proper transfer setup

- **Preparing the gel for transfer**
  - For Southerns, DNA should be depurinated (nicked) w/ HCl to facilitate sticking to membrane , base denatured w/ NaOH to make it single stranded, and neutralized afterwards
  - For Northerns, gel should be soaked in transfer buffer to remove formaldehyde (it inhibits transfer)
- Transfer buffer – 20X SSC (NaCl and Na citrate). Especially important if you are NOT using a + charged membrane.

Setting up a transfer – assembly

- Order of assembly from bottom to top:
  - Wick→ inverted gel→membrane→filter paper→paper towels→weight
  - Transfer proceeds via capillary action, ie simple diffusion
  - Correcting for the mirror image – Keeping track of where your samples are!!!



Review gel and transfer setup

Review gel and transfer setup

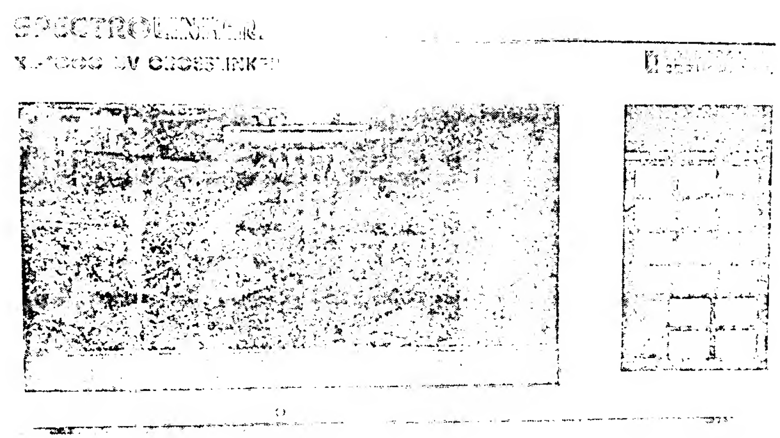
Keys to a successful transfer

- No air bubbles
  - Roll out w/ a pipet
- No short circuits
  - Use parafilm or old xray film to create a "mask"
- Plenty of paper towels
- Time
  - Good to set up just before you leave and let it transfer overnight (or at least 6 hours)

Post transfer issues

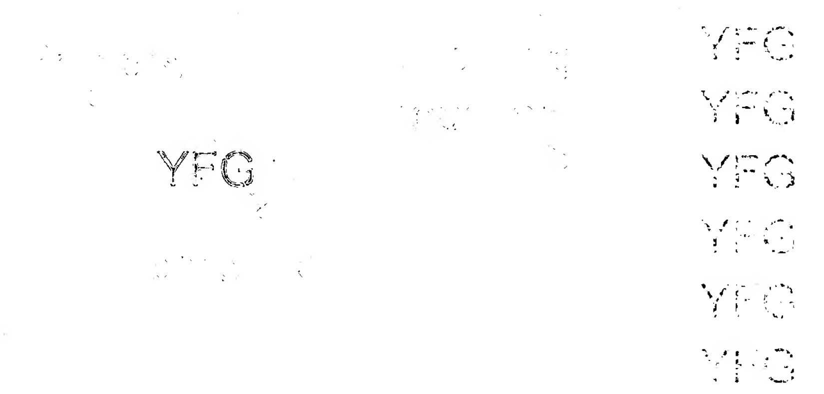
- After a transfer, DNA or RNA is stuck to the membrane, right? WRONG!
- Following transfer, you must immediately fix the sample to the membrane by:
  - Baking (120° for a 30 minutes)
  - UV crosslinking (using a crosslinker)
- After crosslinking, the blot can be stored for years (dry at -80°)

UV crosslinker



Making a probe

- A probe is a copy of a DNA sequence that contains a detectable label

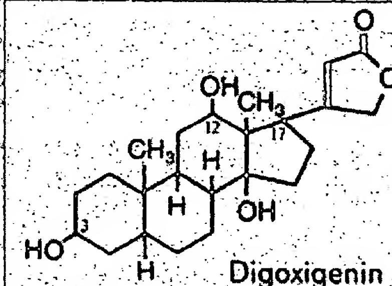


2 Kinds of label

- Non-radioactive
  - Digoxigenin
  - Biotin
- Radioactive
  - ^{32}P

Types of labeling methods

- Non-radioactive
  - Digoxigenin (DIG)
    - The DIG is chemically linked to dUTP – It is incorporated into the random hexamer-primed fragments
    - Use an antibody to detect the DIG after hybridization (just like a "Western" blot



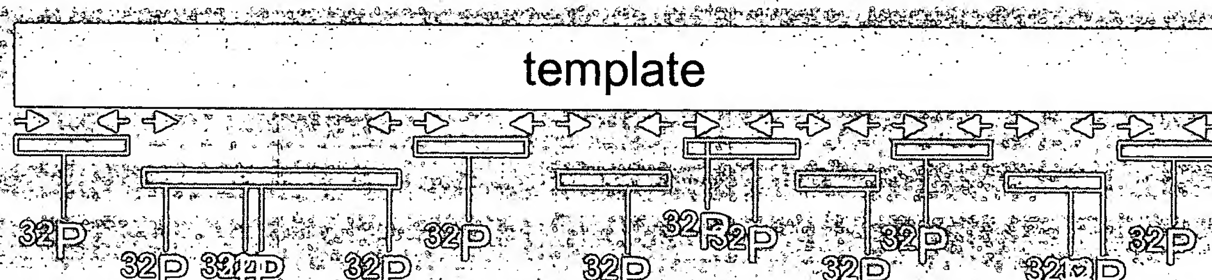
Digoxigenin

  - Advantages
    - Safety
    - Short detection times - usually seconds to minutes
    - Stability – Once made, a probe can be used for a couple of years later
    - Cost – In the long term, very inexpensive compared to other methods
  - Disadvantages
    - Less sensitive
    - Detection requires extra steps
    - Must physically remove probe through a harsh stripping proceedure in order to use the blot again

Types of labeling methods

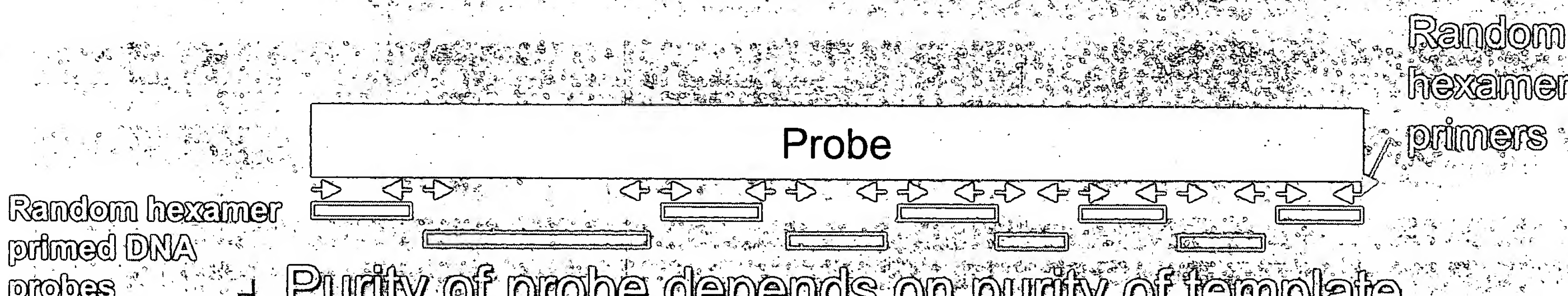
- Radioactive
  - ^{32}P
    - A phosphate in most dCTPs is radiolabeled
    - The radioactive beta particles emitted are detectable with xray film
    - Advantages
      - Sensitivity
      - Ease of reprobing – just let bound probe decay
    - Disadvantages
      - Lots of safety precautions required
      - Unstable
      - Long detection times (sometimes weeks!)
      - Expensive

Random hexamer primed probes that recognize YFG



Making a probe

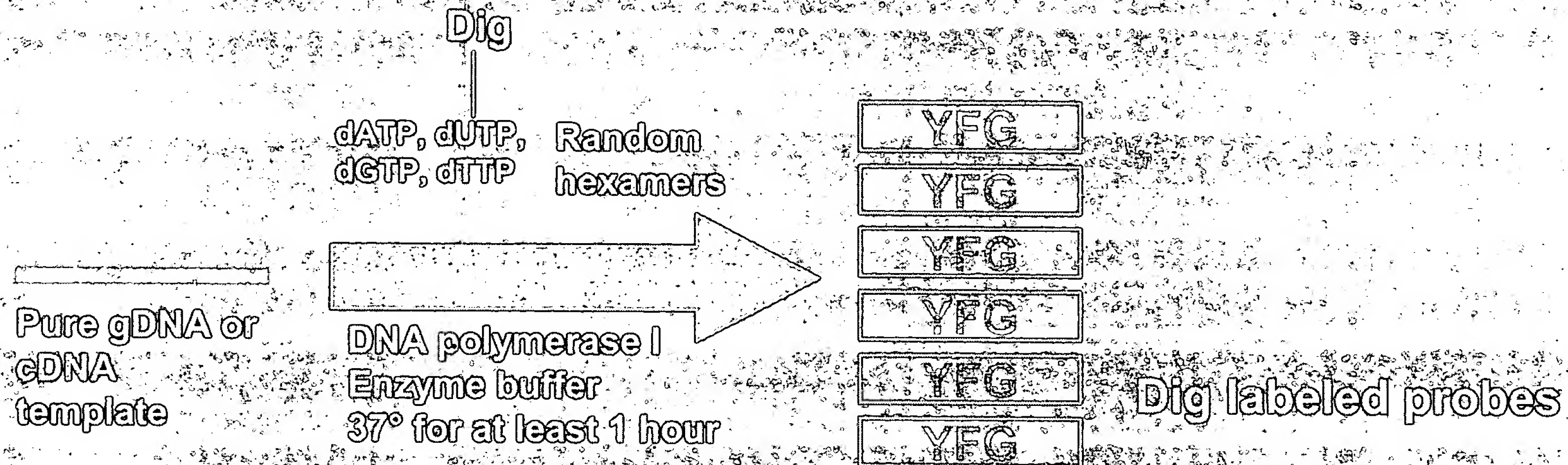
- Commonly used labeling methods
  - Nick translation
  - PCR labeling
  - Random hexamer labeling – easiest and most commonly used



  - Purity of probe depends on purity of template
    - Clean template=pure probe=clean results!

Labeling reaction

- Method for generating 32P labeled and Dig labeled probes is identical



- Purify labeled probes away from unincorporated label by a precipitation step or column filtration
- Quantifying the amount of labeled probe is critical
  - add too much and your blot looks like...
  - add to little and your blot looks like...
- 32P probe - 1-5 ng/ml
- Dig probe – 20-50 ng/ml

Hybridization

Hybridize probe to membrane

- Prehybridization
  - The blot is mostly empty space – your probe will easily bind to this empty space – NOT GOOD!
  - Prehybridization fills this empty space with non-specific nucleic acid – usually salmon sperm DNA (if you're studying salmon or a related fish, you will need to find another blocking agent!)
  - Usually 1 hour at the hybridization temperature is sufficient
  - Hybridization usually goes overnight
  - Usually done in a hybridization oven
- What's in the hybridization solution?
  - Formamide – displaces water; "opens" nucleic acid
  - Denhardts reagent – blocking solution (BSA, PVP, Ficoll)
  - Dextran sulfate – artificially increases probe concentration
  - SDS – alleviates hydrophobic interaction – 2 nucleic acids can stick together simply through hydrophobic interactions

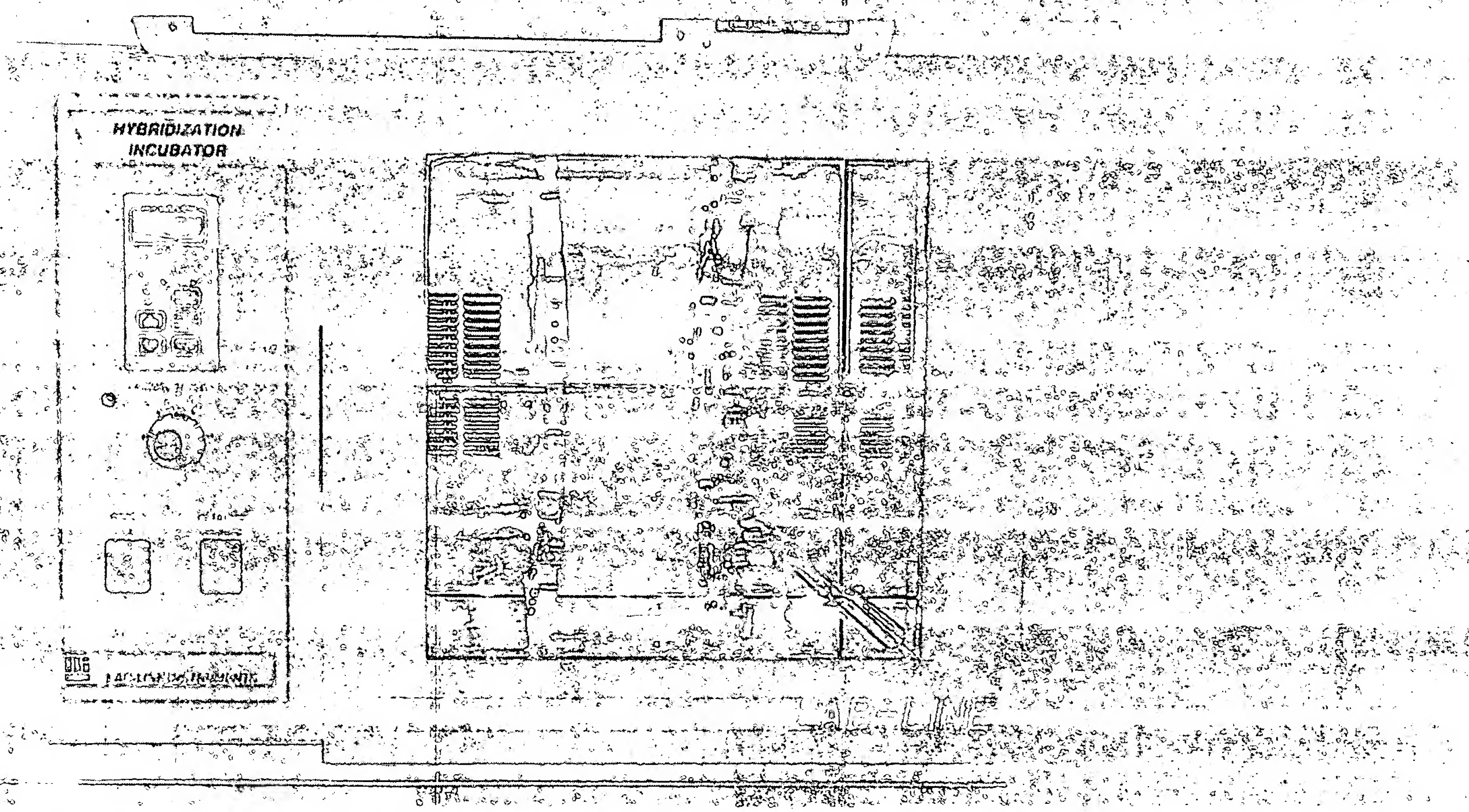
<u>Important points</u>

Hybridization conditions

- Hybridization solutions can be purchased – much easier and more cost effective than making all this stuff yourself!

Once the hybridization has been started, DO NOT let it dry out! Bound probe can not be removed if the blot has dried!

Hybridization oven



Hybridization

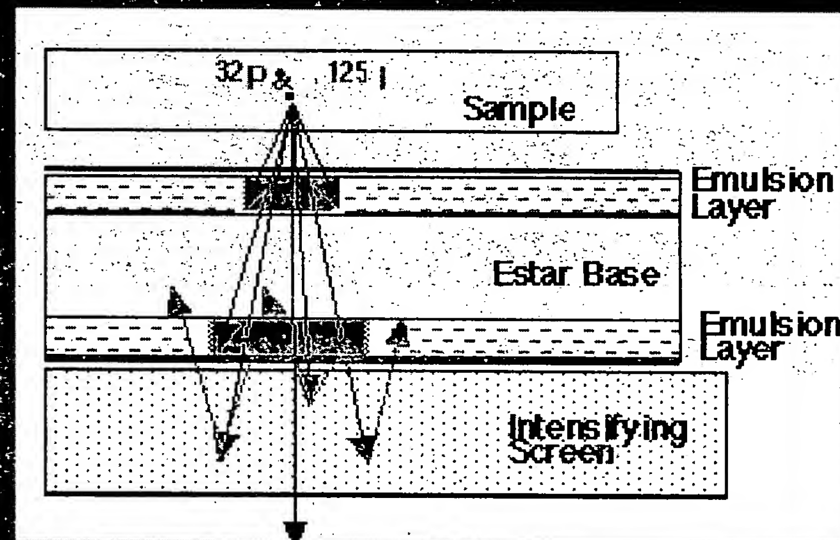
Stringency

- High stringency – Probe won't bind anything
- Low stringency – Probe binds everything
- Things that affect stringency:
  - Salt (NaCl) concentration (lower = high stringency)
  - Formamide concentration (higher = high stringency)
  - Temperature of incubation (higher = high stringency)
- Finding the correct stringency conditions can vary from probe to probe
- Using a commercially available hyb solution, a good place to start is:
  - 68° for an RNA:RNA hybridizations
  - 50° for DNA:RNA hybridizations
  - 42° for DNA:DNA hybridizations

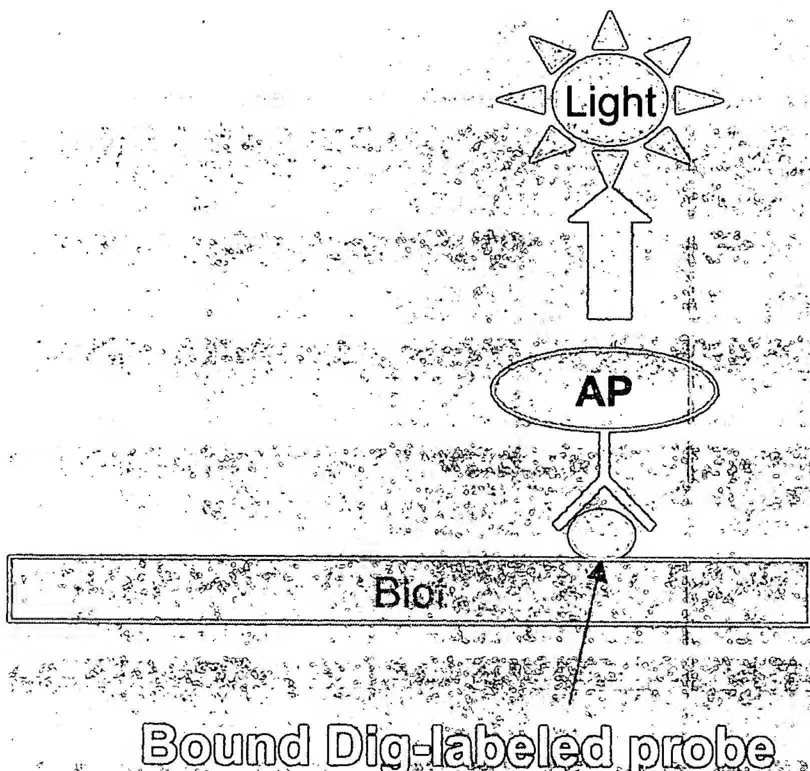
- Post hybridization washes
  - Gets rid of excess unbound probe
  - Contains low salt and high detergent

Detection

- If you used a 32P probe, you can now expose the blot to film with an <u>intensifying screen</u> – amplifies signal at low temperature → put your blot in the -80 freezer.
- Dig probes require a few more steps before you can see them



Detecting the Dig label

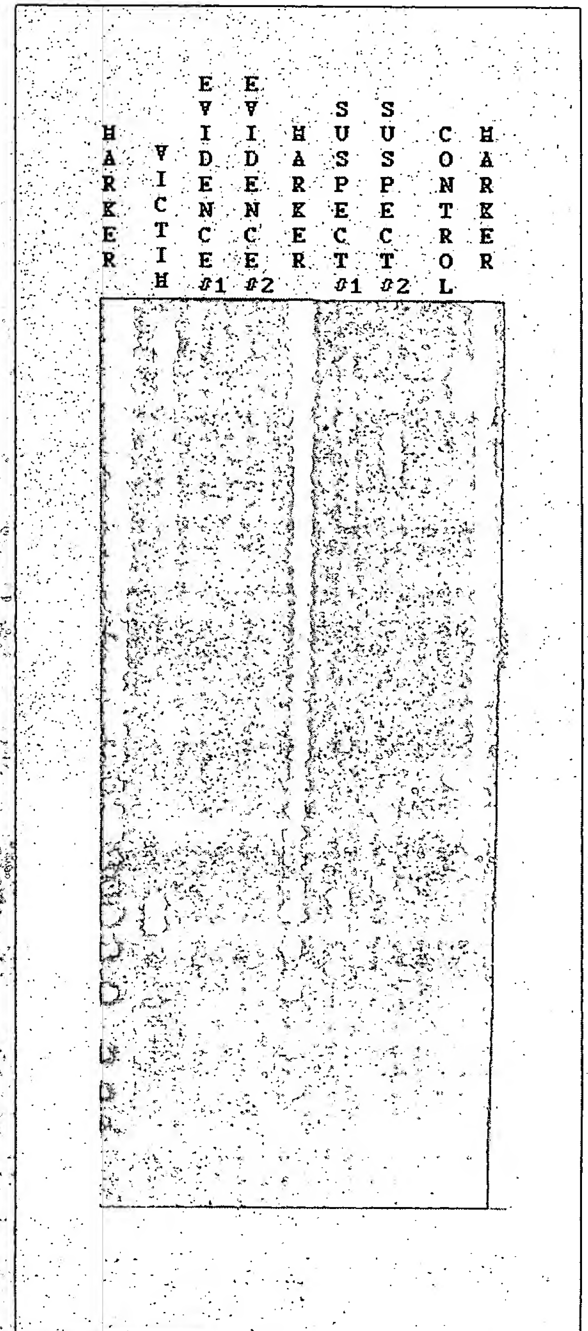


- Anti-Dig antibody coupled to the alkaline phosphatase (AP) enzyme → PROTEIN
- Nylon loves to bind protein
- Block with a BSA solution (30 minutes)
- Incubate w/ antibody (30 minutes)
- Wash (30 minutes)
- AP dephosphorylates AMPPD- Its destabilization gives off 477nm light
- Film detects light
- Detection is done in a film cassette in a dark room

Applications of a Southern blot

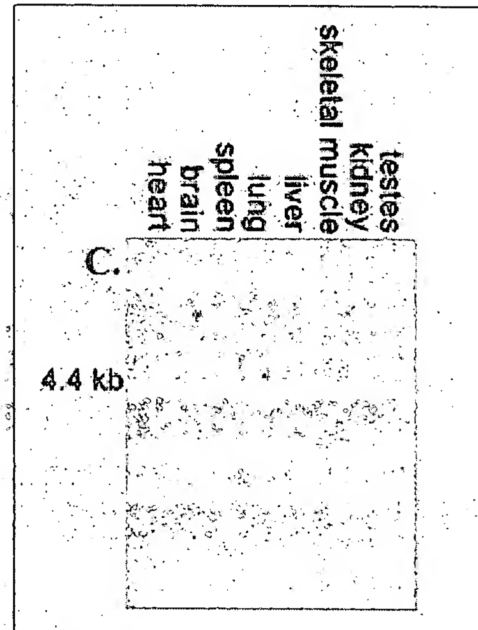
- Genotyping
  - Is your knockout really a knockout?
  - Genetic deficiency mapping
  - SNP mapping (DNA "fingerprinting")
- Variations

FISH

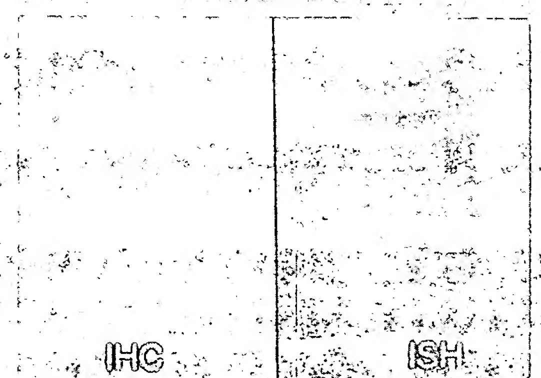


Applications of a Northern blot

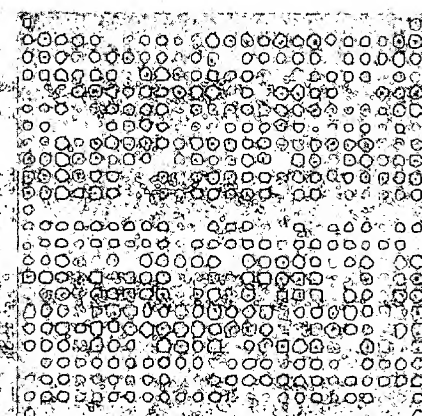
- mRNA detection
  - Where is the gene expressed?
  - Which conditions alter mRNA levels?
  - How big is the gene?
  - Is there more than one isoform?



- Variations



In situ



microarray

Northern interpretation

- Only a Northern will tell you:
  - The size of the message (including all 5’ and 3’ UTR)
  - If there are alternatively spliced isoforms
  - The relative abundance of these isoforms
- Disadvantages
  - Compared to other methods, Northerns are the least sensitive
  - Doesn’t allow cloning for further analysis
  - Only tells you mRNA abundance – doesn’t tell you anything about rate of transcription

Visualizing blots – Summary

- Run samples on a gel
- Transfer to a membrane
- Link sample to membrane
- Make a probe
- Hybridize probe to membrane
- Detect probe

Further ??

- Todd.lamitina@vanderbilt.edu
- Roche biochemicals website
  - http://www.roche-applied-science.com/fst/products.htm?/prod_inf/manuals/dig_man/dig_toc.htm

First Named Inventor	:	Michael E. Spurlock	
Appln. No.	:	09/928,522	
Filed	:	August 13, 2001	Group Art Unit: 1647
Title	:	Bovine Leptin Protein, Antisense and Antibody	Examiner: C. J. Saoud
Docket No.	:	LL31.12-0015	

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of

DECLARATION

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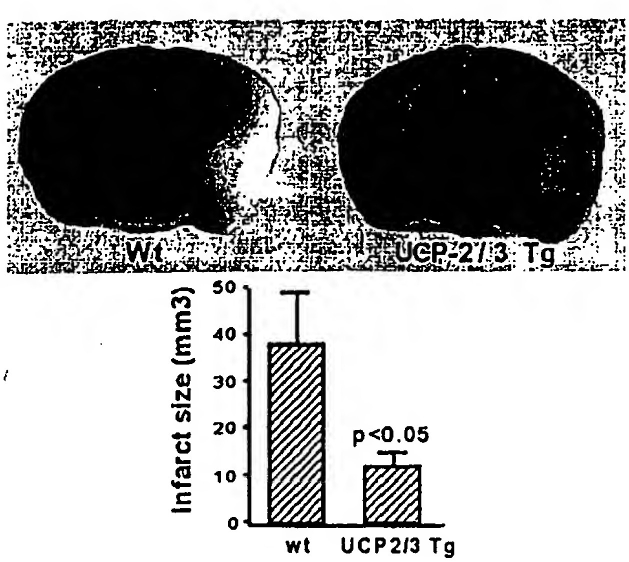
(54) Title: METHODS OF DIAGNOSING, PREVENTING AND TREATING NEUROLOGICAL DISORDERS AND NEURONAL INJURIES



(57) Abstract: The present invention identifies a gene whose gene product provides a protective effect against neurological disorders or neuronal injuries. Further, the invention provides methods for diagnosing or assessing an individual's susceptibility to a neuronal injury such as stroke. Also provided are therapeutic methods for treating patients, and methods for prophylactically treating individuals susceptible to various neurological disorders or neuronal injuries. Additionally, the invention describes screening methods for identifying agents that can be administered to treat individuals that have suffered or are at risk to suffer such disorders or injuries.

WO 02/36829 A2

METHODS OF DIAGNOSING, PREVENTING AND TREATING NEUROLOGICAL DISORDERS AND NEURONAL INJURIES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of priority from U.S. Provisional Patent Application Serial No. 60/244,946, filed November 1, 2000, the full disclosures of which are incorporated herein by reference in their entirety for all purposes.

TECHNICAL FIELD

The present invention relates to methods for diagnosing or assessing an individual's susceptibility to a neurological disorder or a neuronal injury. The invention also relates to therapeutic methods for treating an individual suffering from a neurological disorder or a neuronal injury and methods for identifying agents that can be administered to treat such an individual.

BACKGROUND OF THE INVENTION

Uncoupling proteins (UCPs; thermogenins) are proton-translocating proteins located in the inner mitochondrial membrane that play a role in metabolic processes, particularly non-shivering thermogenesis. The first UCP (UCP-1) was found to be localized in the brown adipose tissue, specialized fat cells that function in heat generation and energy balance. Hibernating and cold-adapted animals have significant stores of such tissue. The evidence indicates that UCPs function to maintain the core body temperature of hibernating mammals and other cold-adapted animals by raising the resting metabolic rate of the animals (see, e.g., Nicolls, D.G. and Locke, R.M. (1984) Physiol. Rev. 64:2-40; and Rothwell, N.J., and Stock, M. J. (1979) Nature 281:31-35).

As the name indicates, UCPs serve an uncoupling function, specifically by uncoupling proton flux through the mitochondrial membranes and ATP synthesis. The mitochondrial oxidation of metabolites (e.g., pyruvate and fatty acids) is accompanied by proton transport out of the mitochondrial matrix, thereby generating a transmembrane proton gradient. The protons re-enter the mitochondria through the protein ATP synthase and drive the synthesis of ATP. The UCPs, however, provide a route for the re-entry of the protons that is uncoupled to ATP synthesis. Consequently, instead of the proton gradient resulting in the

generation of ATP, UCPs act to covert the proton gradient into heat energy and increase the rate of respiration. Exposure to the cold triggers the neural and hormonal stimulation of brown adipose tissue, which in turn increases UCP-mediated proton transport and heat production (see, e.g., Susulic, V.S., and Lowell, B.B. (1996) Curr. Opin. in Endocrinol. and Meta. 3:44-50). Studies conducted with various transgenic models have demonstrated that a reduction in UCP activity correlates with the development of obesity and diabetes (see, e.g., Lowell, B.B., et al. (1993) Nature 366:740; and Kopecky, J. et al. (1995) J. Clin. Invest. 96:2914-23).

While humans have a UCP-1 gene that is active in brown fat, these fat deposits disappear shortly after birth (see, e.g., Bouillaud, et al. (1985) Proc. Natl. Acad. Sci. 82:445-448). Nonetheless, measurements showing that 25% to 30% of the oxygen that humans and other animals utilize to metabolize their food is used to compensate for mitochondrial proton leaks suggested the presence of other UCPs in humans. In fact, several human UCPs have now been identified.

One such UCP is referred to in the literature as UCP-2 or UCPH. The gene encoding this protein maps to human chromosome 11 and has been linked to hyperinsulinemia and obesity. UCP-2 is reported to be expressed in various adult tissue, including brain, muscle and fat cells (see, e.g., Fleury, et al. (1997) Nat. Genet. 15:269-272; Tartaglia, et al. PCT Publication No. WO 96/05861; Gimeno, et al. (1997) Diabetes 46:900-906; and Boss, et al. (1997) FEBS Letters 408:39-42). Allelic variants of UCP-2 appear to have been identified. While some UCP-2 proteins have an alanine at position 55 (see, Fleury, supra, and PCT Publication No. WO 00/06087), other UCP-2 proteins have a valine (see, PCT Publication WO 96/05861). At position 219, some UCP-2 proteins have a threonine (see, PCT Publication WO 96/05861 and PCT Publication WO 00/06087), whereas other UCP-2 proteins have an isoleucine (see, Fleury, supra). Methods for screening for allelic variants are discussed in PCT Publication WO 99/48905.

A third human UCP (UCP-3) has also been recently reported. This UCP is preferentially expressed in human skeletal muscle. The gene encoding this particular UCP maps to human chromosome 11, adjacent to the gene for UCP-2. Studies indicate that UCP-3 expression can be regulated by known thermogenic stimuli such as leptin, β-adrenergic agonists and thyroid hormone (see, e.g., PCT publication WO 98/45313; Boss, et al., (1997) FEBS Letters 408:39-42; Vidal-Puig, et al. (1997) J. Biol. Chem. 272:24129-24132; Solanes

et al. (1997) J. Biol. Chem. 272:25433-25436; and Gong, et al. (1997) J. Biol. Chem. 272:24129-24312).

A fourth human UCP (UCP-4) has been identified. This UCP is expressed in a number of different tissues including, brain, heart, pancreas and muscle tissue (see, e.g., PCT Publication WO 00/04037). Another human UCP (UCP5/BMCP1) is most abundantly expressed in the brain, and at lower levels in most peripheral organs (Sanchis, et al. (1998) J. Biol. Chem. 273: 36411, and PCT Publication WO 00/032624).

Because of the role UCPs play in uncoupling the oxidation of metabolites and the storage of the resulting energy in the form of ATP, UCPs have been viewed primarily as targets for controlling a number of weight disorders (e.g., obesity and underweight disorders), as well as related diseases (e.g., diabetes). However, there is a paucity of information regarding other physiological functions of UCP and how UCP can be utilized in other types of applications other than weight-related applications.

SUMMARY OF THE INVENTION

Provided herein are various methods for diagnosing and treating various neurological disorders and neuronal injuries, particularly stroke and ischemic stroke. Methods for screening agents to identify agents useful in treating neurological disorders and injuries are also provided.

More specifically, certain methods involve diagnosing the occurrence of a stroke or assessing a patient's susceptibility to a stroke by detecting in a patient sample an elevated level of UCP-2 expression. In some methods, detection is accomplished by detecting elevated levels of UCP-2 transcript. Other methods involve detecting an elevated level of UCP-2 polypeptide. Elevated levels of UCP-2 polypeptide can be detected using various immunological techniques such as ELISA assays.

Some of the diagnostic methods provided herein involve assessing a patient's risk of having a stroke. Such methods involve comparing the level of UCP-2 expression in a test sample from the patient with a baseline value, wherein an elevated level of UCP-2 expression in the patient sample relative to the baseline indicates that the patient is at risk for stroke. A variety of baseline levels can be utilized in these methods. In some instances the baseline is the level of UCP-2 expression in a patient sample obtained previously. In other

methods, the baseline value is an average value, a mean value or another statistical value for a population of control individuals.

Certain treatment methods provided herein involve treating a subject having or being susceptible to a neurological disorder or a neuronal injury by administering to the subject an effective amount of an agent that increases the activity of UCP-2. The neurological disorders or neuronal injuries that are amenable to the methods include stroke, Parkinson's disease, Huntington's disease, inherited ataxias, motor neuron diseases, Alzheimer's disease, epilepsy and traumatic brain injury. If the subject is susceptible to the neurological disorder or the neuronal injury, the subject is administered a prophylactic amount of the agent prior to occurring of the disorder or the injury. If, however, the subject has already suffered the neurological disorder or the neuronal injury, then the subject is administered a therapeutic amount of the agent. The agent which increases the activity of UCP-2 can be co-administered with various other agents, including, for example, agents that increase permeability of the blood/brain barrier and/or blood anticoagulants. In certain treatment methods, the agent is a purified UCP-2 polypeptide administered with a pharmaceutically acceptable carrier.

Certain treatment methods involve administering agents that stimulate the synthesis or expression of UCP-2 or a UCP-2 inducing agent. In some methods, the agent administered is a nucleic acid that encodes UCP-2 or a UCP-2 inducer. In such instances, the nucleic acid can be inserted into a viral vector or other expression vectors. The viral vector can also include a promoter operably linked to the nucleic acid which selectively drives expression in nerve cells. The promoter can be a UCP-2 promoter or a heterologous promoter. In certain methods, the viral vector is introduced into the cerebrospinal fluid; in other methods, the vector is injected into the intraventricular space. Still other treatment methods also involve producing ex vivo genetically-modified neuronal or non-neuronal stem cells that harbor the vector that includes a nucleic acid encoding UCP-2. The modified stem cells are then introduced into the intracerebroventricular space or into the cerebrospinal fluid.

A variety of screening methods is provided. Certain of these methods involve screening for an agent useful for treating a neuronal injury (e.g., stroke, traumatic brain injury) or a neurological disorder (e.g., Parkinson's disease, Alzheimer's disease, or epilepsy) by identifying an agent that upregulates UCP-2 expression and/or activity. Some of the screening methods involve: (a) administering to a test subject a test compound, wherein the

test subject is a mammal other than a human; (b) preconditioning the test subject; and (c) determining in a sample from the test subject the expression level of UCP-2 to identify a test agent that upregulates UCP-2 expression in the test subject.

In other screening methods, agents useful for treating a neurological disorder or a neuronal injury are identified by identifying an agent that inhibits cellular apoptosis. Often such methods are conducted to identify agents useful in treating stroke or ischemic stroke. Certain screens identify compounds that inhibit the loss of mitochondrial membrane potential. Other screens provided herein identify agents that inhibit opening of the mitochondrial transition pore and release of cytochrome c from mitochondria and/or agents that inhibit the activation of caspases, as these events are associated with cellular apoptosis.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A-1D are photomicrographs showing the results of in-situ hybridization experiments conducted with brain sections obtained from rats subjected to various treatment protocols which show the regions of the brain in which UCP-2 mRNA is localized. In particular, the photographs show the localization of a labeled probe (oligo 28) that specifically hybridizes with UCP-2 at the CA1, CA3 and DG regions of the rat brain hippocampus. FIG. 1A shows that UCP-2 expression is low in the CA1 under normal conditions. FIG. 1B illustrates the increase in UCP-2 mRNA levels at two days following a 3 minute ischemic event. FIG. 1C shows that there is no increase in UCP-2 mRNA levels at one day of recovery after a 10 minute ischemic insult. FIG. 1D shows that UCP-2 mRNA levels are increased at 24 h after 3 minutes of ischemic preconditioning prior to the 10 minute ischemic insult (3 min – 24 h – 10 min – 24 h-in situ).

FIGS. 2A-2D are photomicrographs that illustrate the same events illustrated in FIGS. 1A-1D but utilizing a different labeled probe (oligo 33) that specifically hybridizes to UCP-2 mRNA.

FIG. 3 shows western blot analysis of UCP-2 protein levels in rat primary cortical neuronal cultures preconditioned by exposure to OGD for 10 min or to 40μM NMDA for 5 min.

FIG. 4 shows western blot analysis of overexpression of recombinant UCP-2 in rat primary neuronal cultures by infection of neurons with an adenovirus containing a nucleic acid encoding UCP-2.

FIG.5 shows immunofluorescence analysis of overexpression of recombinant UCP-2 in rat primary neuronal cultures.

FIGS. 6A and 6B are plots illustrating the neuroprotective effect obtained by infection of neurons with an adenovirus containing a nucleic acid encoding UCP-2. Specifically, the plot illustrates protection of neuronal cultures against cell death induced by combined oxygen-glucose deprivation (OGD). FIG. 6A is a plot of cell death for uninfected cells (No Ad), control cells infected with an adenovirus bearing the lacZ gene (Ad.LacZ) instead of the UCP-2 gene or test cells infected with an adenovirus having the UCP-2 gene (Ad.UCP2). Tests were conducted either without OGD (open boxes) or 90 minutes of OGD (darkened boxes). FIG. 6B shows the extent of cell death for cell cultures not subjected to OGD (control) and cell cultures exposed to 10 min OGD, 90 min OGD or 10 minute of OGD preconditioning followed by 90 min of OGD.

FIG. 7 shows effects of UCP-2 overexpression on ischemic brain damage *in vivo*.

FIG. 8 shows flow cytometry analysis of mitochondria isolated from rat brain cultures.

FIG. 9 shows results of western blot analysis of cytochrome c protein levels in cytosolic and mitochondrial cell fractions.

FIG. 10 is a chart showing the effect of UCP-2 expression on caspase 3 activation, with caspase 3 being induced by subjecting cell cultures to 90 min of OGD. Tests were performed with cells infected with an adenovirus having the lacZ gene instead of UCP-2 (Ad.LacZ; control) and cells infected with an adenovirus including the UCP-2 gene (Ad.UCP2).

DETAILED DESCRIPTION

I. Definitions

As used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural references unless the content clearly dictates otherwise.

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention belongs. The following references provide one of skill with a general definition of many of the terms used in this invention: Singleton *et al.*, DICTIONARY OF MICROBIOLOGY

AND MOLECULAR BIOLOGY (2d ed. 1994); THE CAMBRIDGE DICTIONARY OF SCIENCE AND TECHNOLOGY (Walker ed., 1988); THE GLOSSARY OF GENETICS, 5TH ED., R. Rieger et al. (eds.), Springer Verlag (1991); and Hale & Marham, THE HARPER COLLINS DICTIONARY OF BIOLOGY (1991). As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

Various biochemical and molecular biology methods are well known in the art. For example, methods of isolation and purification of nucleic acids are described in detail in WO 97/10365, WO 97/27317, Chapter 3 of Laboratory Techniques in Biochemistry and Molecular Biology: Hybridization With Nucleic Acid Probes, Part I. Theory and Nucleic Acid Preparation, (P. Tijssen, ed.) Elsevier, N.Y. (1993); Chapter 3 of Laboratory Techniques in Biochemistry and Molecular Biology: Hybridization With Nucleic Acid Probes, Part 1. Theory and Nucleic Acid Preparation, (P. Tijssen, ed.) Elsevier, N.Y. (1993); and Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, N.Y., (1989); Current Protocols in Molecular Biology, (Ausubel, F.M. et al., eds.) John Wiley & Sons, Inc., New York (1987-1993). Large numbers of tissue samples can be readily processed using techniques known in the art, including, for example, the single-step RNA isolation process of Chomczynski, P. described in U.S. Pat. No. 4,843,155.

A variety of methods are known for amplifying nucleic acids. Examples of suitable amplification techniques include, but are not limited to: (1) the polymerase chain reaction (PCR) [see, e.g., PCR Technology: Principles and Applications for DNA Amplification (H.A. Erlich, Ed.) Freeman Press, NY, NY (1992); PCR Protocols: A Guide to Methods and Applications (Innis, et al., Eds.) Academic Press, San Diego, CA (1990); and U.S. Patent Nos. 4,683,202 and 4,683,195]; (2) the ligase chain reaction (LCR) [see, e.g., Wu and Wallace, Genomics 4:560 (1989) and Landegren et al., Science 241:1077 (1988)]; (3) transcription amplification [see, e.g., Kwoh et al., Proc. Natl. Acad. Sci. USA 86:1173 (1989)]; (4) self-sustained sequence replication [see, e.g., Guatelli et al., Proc. Natl. Acad. Sci. USA, 87:1874 (1990)]; and, (5) nucleic acid based sequence amplification (NABSA) [see, e.g., Sooknanan, R. and Malek, L., BioTechnology 13: 563-65 (1995)].

The terms "nucleic acid," "polynucleotide," and "oligonucleotide" are used herein to include a polymeric form of nucleotides of any length, including, but not limited to, ribonucleotides or deoxyribonucleotides. There is no intended distinction in length between these terms. Further, these terms refer only to the primary structure of the molecule. Thus, in

certain embodiments these terms can include triple-, double- and single-stranded DNA, as well as triple-, double- and single-stranded RNA. They also include modifications, such as by methylation and/or by capping, and unmodified forms of the polynucleotide. More particularly, the terms "nucleic acid," "polynucleotide," and "oligonucleotide," include polydeoxyribonucleotides (containing 2-deoxy-D-ribose), polyribonucleotides (containing D-ribose), any other type of polynucleotide which is an N– or C-glycoside of a purine or pyrimidine base, and other polymers containing nonnucleotidic backbones, for example, polyamide (*e.g.*, peptide nucleic acids (PNAs)) and polymorpholino (commercially available from the Anti-Virals, Inc., Corvallis, Oregon, as Neugene) polymers, and other synthetic sequence-specific nucleic acid polymers providing that the polymers contain nucleobases in a configuration which allows for base pairing and base stacking, such as is found in DNA and RNA. Thus, in certain embodiments these terms include, for example, 3'-deoxy-2',5'-DNA, oligodeoxyribonucleotide N3' P5' phosphoramidates, 2'-O-alkyl-substituted RNA, double- and single-stranded DNA, as well as double- and single-stranded RNA, DNA:RNA hybrids, and hybrids between PNAs and DNA or RNA, and also include known types of modifications, for example, labels that are known in the art, methylation, "caps," substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages *(e.g.*, methyl phosphonates, phosphotriesters, phosphoramidates, carbamates, etc.), with negatively charged linkages (*e.g.*, phosphorothioates, phosphorodithioates), and with positively charged linkages (*e.g.*, aminoalklyphosphoramidates, aminoalkylphosphotriesters), those containing pendant moieties, such as, for example, proteins (including nucleases, toxins, antibodies, signal peptides, poly-L-lysine), those with intercalators (*e.g.*, acridine, psoralen), those containing chelators (*e.g.*, metals, radioactive metals, boron, oxidative metals), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids), as well as unmodified forms of the polynucleotide or oligonucleotide. In particular, DNA is deoxyribonucleic acid.

A "probe" is an nucleic acid capable of binding to a target nucleic acid of complementary sequence through one or more types of chemical bonds, usually through complementary base pairing, usually through hydrogen bond formation, thus forming a duplex structure. The probe binds or hybridizes to a "probe binding site." The probe can be labeled with a detectable label to permit facile detection of the probe, particularly once the

probe has hybridized to its complementary target. The label attached to the probe can include any of a variety of different labels known in the art that can be detected by chemical or physical means, for example. Suitable labels that can be attached to probes include, but are not limited to, radioisotopes, fluorophores, chromophores, mass labels, electron dense particles, magnetic particles, spin labels, molecules that emit chemiluminescence, electrochemically active molecules, enzymes, cofactors, and enzyme substrates. Probes can vary significantly in size. Some probes are relatively short. Generally, probes are at least 7 to 15 nucleotides in length. Other probes are at least 20, 30 or 40 nucleotides long. Still other probes are somewhat longer, being at least 50, 60, 70, 80, 90 nucleotides long. Yet other probes are longer still, and are at least 100, 150, 200 or more nucleotides long. Probes can be of any specific length that falls within the foregoing ranges as well.

A "primer" is a single-stranded polynucleotide capable of acting as a point of initiation of template-directed DNA synthesis under appropriate conditions (*i.e.*, in the presence of four different nucleoside triphosphates and an agent for polymerization, such as, DNA or RNA polymerase or reverse transcriptase) in an appropriate buffer and at a suitable temperature. The appropriate length of a primer depends on the intended use of the primer but typically is at least 7 nucleotides long and, more typically range from 10 to 30 nucleotides in length. Other primers can be somewhat longer such as 30 to 50 nucleotides long. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with the template. A primer need not reflect the exact sequence of the template but must be sufficiently complementary to hybridize with a template. The term "primer site" or "primer binding site" refers to the segment of the target DNA to which a primer hybridizes. The term "primer pair" means a set of primers including a 5' "upstream primer" that hybridizes with the complement of the 5' end of the DNA sequence to be amplified and a 3' "downstream primer" that hybridizes with the 3' end of the sequence to be amplified.

A primer that is "perfectly complementary" has a sequence fully complementary across the entire length of the primer and has no mismatches. The primer is typically perfectly complementary to a portion (subsequence) of a target sequence. A "mismatch" refers to a site at which the nucleotide in the primer and the nucleotide in the target nucleic acid with which it is aligned are not complementary. The term "substantially complementary" when used in reference to a primer means that a primer is not perfectly

complementary to its target sequence; instead, the primer is only sufficiently complementary to hybridize selectively to its respective strand at the desired primer-binding site.

The term "complementary" means that one nucleic acid is identical to, or hybridizes selectively to, another nucleic acid molecule. Selectivity of hybridization exists when hybridization occurs that is more selective than total lack of specificity. Typically, selective hybridization will occur when there is at least about 55% identity over a stretch of at least 14-25 nucleotides, preferably at least 65%, more preferably at least 75%, and most preferably at least 90%. Preferably, one nucleic acid hybridizes specifically to the other nucleic acid. See M. Kanehisa, *Nucleic Acids Res. 12:203 (1984).*

The term "operably linked" refers to functional linkage between a nucleic acid expression control sequence (such as a promoter, signal sequence, or array of transcription factor binding sites) and a second polynucleotide, wherein the expression control sequence affects transcription and/or translation of the second polynucleotide.

A "heterologous sequence" or a "heterologous nucleic acid," as used herein, is one that originates from a source foreign to the particular host cell, or, if from the same source, is modified from its original form. Thus, a heterologous gene in a prokaryotic host cell includes a gene that, although being endogenous to the particular host cell, has been modified. Modification of the heterologous sequence can occur, *e.g.*, by treating the DNA with a restriction enzyme to generate a DNA fragment that is capable of being operably linked to the promoter. Techniques such as site-directed mutagenesis are also useful for modifying a heterologous nucleic acid.

The term "recombinant" when used with reference to a cell indicates that the cell replicates a heterologous nucleic acid, or expresses a peptide or protein encoded by a heterologous nucleic acid. Recombinant cells can contain genes that are not found within the native (non-recombinant) form of the cell. Recombinant cells can also contain genes found in the native form of the cell wherein the genes are modified and re-introduced into the cell by artificial means. The term also encompasses cells that contain a nucleic acid endogenous to the cell that has been modified without removing the nucleic acid from the cell; such modifications include those obtained by gene replacement, site-specific mutation, and related techniques.

A "recombinant expression cassette" or simply an "expression cassette" is a nucleic acid construct, generated recombinantly or synthetically, that has control elements

that are capable of affecting expression of a structural gene that is operably linked to the control elements in hosts compatible with such sequences. Expression cassettes include at least promoters and optionally, transcription termination signals. Typically, the recombinant expression cassette includes at least a nucleic acid to be transcribed (*e.g.*, a nucleic acid encoding UCP-2) and a promoter. Additional factors necessary or helpful in effecting expression can also be used as described herein. For example, transcription termination signals, enhancers, and other nucleic acid sequences that influence gene expression, can also be included in an expression cassette.

The term "stringent conditions" refers to conditions under which a probe or primer will hybridize to its target subsequence, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. Generally, stringent conditions are selected to be about 5 °C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength and pH. In other instances, stringent conditions are chosen to be about 20 °C or 25 °C below the melting temperature of the sequence and a probe with exact or nearly exact complementarity to the target. As used herein, the melting temperature is the temperature at which a population of double-stranded nucleic acid molecules becomes half-dissociated into single strands. Methods for calculating the T_m of nucleic acids are well known in the art (see, *e.g.*, Berger and Kimmel (1987) Methods in Enzymology, vol. 152: Guide to Molecular Cloning Techniques, San Diego: Academic Press, Inc. and Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., vols. 1-3, Cold Spring Harbor Laboratory), both incorporated herein by reference. As indicated by standard references, a simple estimate of the T_m value can be calculated by the equation: $T_m = 81.5 + 0.41(\% G + C)$, when a nucleic acid is in aqueous solution at 1 M NaCl (*see* e.g., Anderson and Young, "Quantitative Filter Hybridization," in *Nucleic Acid Hybridization* (1985)). Other references include more sophisticated computations which take structural as well as sequence characteristics into account for the calculation of T_m. The melting temperature of a hybrid (and thus the conditions for stringent hybridization) is affected by various factors such as the length and nature (DNA, RNA, base composition) of the probe and nature of the target (DNA, RNA, base composition, present in solution or immobilized, and the like), and the concentration of salts and other components (*e.g.*, the presence or absence of formamide, dextran sulfate, polyethylene glycol). The effects of these factors are well known and are

discussed in standard references in the art, see *e.g.*, Sambrook, *supra*, and Ausubel, *supra*. Typically, stringent conditions will be those in which the salt concentration is less than about 1.0 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30 °C for short probes or primers (e.g., 10 to 50 nucleotides) and at least about 60 °C for long probes or primers (e.g., greater than 50 nucleotides). Stringent conditions can also be achieved with the addition of destabilizing agents such as formamide.

The term "expression" when used in the context of expression or a gene or nucleic acid refers to the conversion of the information, contained in a gene, into a gene product. A gene product can be the direct transcriptional product of a gene (*e.g.*, mRNA, tRNA, rRNA, antisense RNA, ribozyme, structural RNA or any other type of RNA) or a protein produced by translation of a mRNA. Gene products also include RNAs which are modified, by processes such as capping, polyadenylation, methylation, and editing, and proteins modified by, for example, methylation, acetylation, phosphorylation, ubiquitination, ADP-ribosylation, myristilation, and glycosylation.

The terms "upregulated and "activation" when used in reference to the expression of a nucleic acid such as a gene (particularly UCP-2) refers to any process which results in an increase in production of a gene product. A gene product can be either RNA (including, but not limited to, mRNA, rRNA, tRNA, and structural RNA) or protein. Accordingly, gene upregulation or activation includes those processes that increase transcription of a gene and/or translation of a mRNA. Examples of gene upregulation or activation processes that increase transcription include, but are not limited to, those that facilitate formation of a transcription initiation complex, those that increase transcription initiation rate, those that increase transcription elongation rate, those that increase processivity of transcription and those that relieve transcriptional repression (by, for example, blocking the binding of a transcriptional repressor). Gene upregulation or activation can constitute, for example, inhibition of repression as well as stimulation of expression above an existing level. Examples of gene upregulation or activation processes that increase translation include those that increase translational initiation, those that increase translational elongation and those that increase mRNA stability.

The level of gene expression, including the level of gene activation or upregulation, can be quantitated utilizing a number of established techniques including, but

not limited to, Northern-Blots, RNase protection assays (RPA), nucleic acid probe arrays, quantitative PCR (e.g., the so-called TaqMan assays), dot blot assays and in-situ hybridization. These are described further infra.

In general, gene upregulation or activation comprises any detectable increase in the production of a gene product, preferably an increase in production of a gene product by at least 50 to 100%, in other instances from about 2- to about 5-fold or any integer therebetween, in still other instances between about 5- and about 10-fold or any integer therebetween, sometimes between about 10- and about 20-fold or any integer therebetween, in other instances between about 20- and about 50-fold or any integer therebetween, in yet other instances between about 50- and about 100-fold or any integer therebetween, and in still other instances 100-fold or more. The terms upregulated and gene activation can also mean that the observed activity relative to a baseline level is a is a statistically significant difference (i.e., increase).

As used herein a "baseline value" generally refers to a value (or ranges of values) against which an experimental or determined value (e.g., one determined for a patient sample as part of a diagnostic or prognostic test) is compared.. Thus, in the case of UCP-2 upregulation, the baseline value can be a value for UCP-2 activity or expression for a sample obtained from the same individual at a different time point. In other instances, the baseline value is a value determined for a control cell or individual, or a statistical value (e.g., an average or mean) established for a population of control cells or individuals. In the specific instance of UCP-2 upregulation, the control can be a cell, individual or populations thereof for which UCP-2 levels would not be expected to be upregulated. Thus, for instance, a control individual or control population can include healthy individuals, particularly those that have not suffered a stroke or those not susceptible to stroke. The population that serves as a control can vary in size, having as few as a single member, but potentially including tens, hundreds, thousands, tens of thousands or more individuals. When the control is a large population, the baseline value can be a statistical value determined from individual values for each member or a value determined from the control population as an aggregate (e.g., a value measured for a population of cells within a well).

A difference is typically considered to be "statistically significant" if the probability of the observed difference occurring by chance (the p-value) is less than some predetermined level. As used herein a "statistically significant difference" refers to a p-value

that is < 0.05, preferably < 0.01 and most preferably < 0.001. Typically, the upregulation of UCP-2 is at least 20%, in still other instances at least 40% or 50%, in yet other instances at least 70% or 80%, and in other instances at least 90% or 100%, although the change can be considerably higher.

"Polypeptide" and "protein" are used interchangeably herein and include a molecular chain of amino acids linked through peptide bonds. The terms do not refer to a specific length of the product. Thus, "peptides," "oligopeptides," and "proteins" are included within the definition of polypeptide. The terms include post-translational modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like. In addition, protein fragments, analogs, mutated or variant proteins, fusion proteins and the like are included within the meaning of polypeptide.

For the sake of simplicity, as used herein, the term "UCP-2 polypeptide," "UCP-2 protein" or simply "UCP-2" refers to a protein having a native UCP-2 amino acid sequence, as well as variants and modified forms regardless of origin or mode of preparation. The UCP-2 protein can be from any animal source, typically a mammalian source, most typically a human. A UCP-2 protein having a native amino acid sequence is a protein having the same amino acid sequence as a UCP-2 as obtained from nature (i.e., a naturally occurring UCP-2). Such native sequence UCP-2 proteins can be isolated from nature or can be prepared using standard recombinant and/or synthetic methods. Native sequence UCP-2 proteins specifically encompass naturally occurring truncated or soluble forms, naturally occurring variant forms (e.g., alternatively spliced forms), naturally occurring allelic variants of UCP-2 and forms including postranslational modifications. One specific example of a native sequence of UCP-2 is the full-length native sequence UCP-2 comprising the amino acid residues set forth as SEQ ID NO:2 (Genbank U76367), as reported by Fleury et al. (1997) Nature Genetics 15:269-272, which is incorporated by reference in its entirety. This protein is encoded by the a nucleic acid having the sequence set forth in SEQ ID NO:1. Other native sequence UCP-2 proteins have the same sequence as set forth in SEQ ID NO:2, except that amino residue 55 is valine instead of alanine (see, Tartaglia, et al., PCT Publication No. WO 96/05861, which is incorporated by reference) and/or amino acid residue 219 is threonine instead of isoleucine (see, Tartaglia, supra, and Chen, et al., PCT Publication WO 00/06087, which is incorporated by reference). A native sequence UCP-2 protein

includes proteins following post-translational modifications such as glycosylation of certain amino acid residues.

UCP-2 variants refer to proteins that are functional equivalents to a native sequence UCP-2 protein that have similar amino acid sequences and retain, to some extent, one of the UCP-2 activities. Variants also include fragments that retain UCP-2 activity. UCP-2 activities include, but are not limited to, uncoupling activity and immunological cross-reactivity with antibodies that specifically bind to native sequence UCP-2. Preferred functional equivalents retain all of the activities of UCP-2, although the activity of such equivalent proteins can be stronger or weaker when compared on a quantitative basis. Typically, functional equivalents have activities that are within 1% to 10,000% of the activity of a native sequence UCP-2, while other functional equivalents have activities that are 10% to 1000%, or 50% to 500% of that of a native sequence UCP-2.

Variants also include proteins that are substantially identical to a native sequence UCP-2. Such variants include proteins having amino acid alterations such as deletions, insertions and/or substitutions. A "deletion" refers to the absence of one or more amino acid residues in the related protein. The term "insertion" refers to the addition of one or more amino acids in the related protein. A "substitution" refers to the replacement of one or more amino acid residues by another amino acid residue in the polypeptide. Typically, such alterations are conservative in nature such that the activity of the variant protein is substantially similar to a native sequence UCP-2 (see, e.g., Creighton (1984) *Proteins*, W.H. Freeman and Company). In the case of substitutions, the amino acid replacing another amino acid usually has similar structural and/or chemical properties. Insertions and deletions are typically in the range of 1 to 5 amino acids, although depending upon the location of the insertion, more amino acids can be inserted or removed. The variations can be made using methods known in the art such as site-directed mutagenesis (Carter, et al. (1986) Nucl. Acids Res. 13:4331; Zoller et al. (1987) Nucl. Acids Res. 10:6487), cassette mutagenesis (Wells et al. (1985) Gene 34:315), restriction selection mutagenesis (Wells, et al. (1986) Philos. Trans. R. Soc. London SerA 317:415), and PCR mutagenesis (Sambrook, et al. (1989) Molecular Cloning, Cold Spring Harbor Laboratory Press).

Modified forms of UCP-2 generally refer to proteins in which one or more amino acids of a native sequence UCP-2 have been altered to a non-naturally occurring amino acid residue. Such modifications can occur during or after translation and include, but

are not limited to, phosphorylation, glycosylation, cross-linking, acylation and proteolytic cleavage.

In view of the foregoing, references to a "UCP-2 nucleic acid" includes nucleic acids that encode for the various UCP-2 proteins described supra. The UCP-2 nucleic acids include nucleic acids (e.g., DNA and RNA) that are complementary to the coding sequences. Given the degeneracy of the genetic code, UCP-2 nucleotides also include all degenerate sequences that encode for the UCP-2 proteins as defined supra.

The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptides, refer to two or more sequences or subsequences that are the same or have a specified percentage of nucleotides or amino acid residues that are the same, when compared and aligned for maximum correspondence, as measured using a sequence comparison algorithm such as those described below for example, or by visual inspection.

The phrase "substantially identical," in the context of two nucleic acids or polypeptides, refers to two or more sequences or subsequences that have at least 75%, preferably at least 85%, more preferably at least 90%, 95% or higher nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using a sequence comparison algorithm such as those described below for example, or by visual inspection. Preferably, the substantial identity exists over a region of the sequences that is at least about 40-60 residues in length, preferably over a longer region than 60-80 amino acids, more preferably at least about 90-100 residues, and most preferably the sequences are substantially identical over the full length of the sequences being compared, such as the coding region of a nucleotide for example.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444

(1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by visual inspection [*see generally*, Current Protocols in Molecular Biology, (Ausubel, F.M. et al., eds.) John Wiley & Sons, Inc., New York (1987-1999, including supplements such as supplement 46 (April 1999)]. Use of these programs to conduct sequence comparisons are typically conducted using the default parameters specific for each program.

Another example of algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul *et al., J. Mol. Biol.* 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al*, *supra.*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. For identifying whether a nucleic acid or polypeptide is within the scope of the invention, the default parameters of the BLAST programs are suitable. The BLASTN program (for nucleotide sequences) uses as defaults a word length (W) of 11, an expectation (E) of 10, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a word length (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix. The TBLATN program (using protein sequence for nucleotide sequence) uses as defaults a word length (W) of 3, an expectation (E) of 10, and a BLOSUM 62 scoring matrix. (*see* Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)).

In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (*see, e.g.*, Karlin & Altschul, *Proc. Nat'l. Acad. Sci. USA* 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions. "Bind(s) substantially" refers to complementary hybridization between a probe nucleic acid and a target nucleic acid and embraces minor mismatches that can be accommodated by reducing the stringency of the hybridization media to achieve the desired detection of the target polynucleotide sequence. The phrase "hybridizing specifically to", refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent conditions when that sequence is present in a complex mixture (*e.g.*, total cellular) DNA or RNA.

A further indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the polypeptide encoded by the second nucleic acid, as described below.

"Conservatively modified variations" of a particular polynucleotide sequence refers to those polynucleotides that encode identical or essentially identical amino acid sequences, or where the polynucleotide does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given polypeptide. For instance, the codons CGU, CGC, CGA, CGG, AGA, and AGG all encode the amino acid arginine. Thus, at every position where an arginine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of "conservatively modified variations." Every polynucleotide sequence described herein which encodes a

polypeptide also describes every possible silent variation, except where otherwise noted. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine) can be modified to yield a functionally identical molecule by standard techniques. Accordingly, each "silent variation" of a nucleic acid which encodes a polypeptide is implicit in each described sequence.

A polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions. A "conservative substitution," when describing a protein, refers to a change in the amino acid composition of the protein that does not substantially alter the protein's activity. Thus, "conservatively modified variations" of a particular amino acid sequence refers to amino acid substitutions of those amino acids that are not critical for protein activity or substitution of amino acids with other amino acids having similar properties (*e.g.*, acidic, basic, positively or negatively charged, polar or non-polar, *etc.*) such that the substitutions of even critical amino acids do not substantially alter activity. Conservative substitution tables providing functionally similar amino acids are well-known in the art. *See, e.g.*, Creighton (1984) *Proteins*, W.H. Freeman and Company. In addition, individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids in an encoded sequence are also "conservatively modified variations."

The term "antibody" as used herein includes antibodies obtained from both polyclonal and monoclonal preparations, as well as the following: (i) hybrid (chimeric) antibody molecules (see, for example, Winter *et al.* (1991) *Nature* 349:293-299; and U.S. Patent No. 4,816,567); (ii) F(ab')2 and F(ab) fragments; (iii) Fv molecules (noncovalent heterodimers, see, for example, Inbar et al. (1972) Proc. Natl. Acad. Sci. USA 69:2659-2662; and Ehrlich et al. (1980) Biochem 19:4091-4096); (iv) single-chain Fv molecules (sFv) (see, for example, Huston et al. (1988) Proc. Natl. Acad. Sci. USA 85:5879-5883); (v) dimeric and trimeric antibody fragment constructs; (vi) humanized antibody molecules (see, for example, Riechmann et al. (1988) Nature 332:323-327; Verhoeyan et al. (1988) Science 239:1534-1536; and U.K. Patent Publication No. GB 2,276,169, published 21 September 1994); (vii) Mini-antibodies or minibodies (i.e., sFv polypeptide chains that include oligomerization domains at their C-termini, separated from the sFv by a hinge region; see, e.g., Pack et al. (1992) Biochem 31:1579-1584; Cumber et al. (1992) J. Immunology 149B:120-126); and, (vii) any functional fragments obtained from such molecules, wherein such fragments retain

specific-binding properties of the parent antibody molecule.

The phrases "specifically binds" when referring to a protein, "specifically immunologically cross reactive with," or simply "specifically immunoreactive with" when referring to an antibody, refers to a binding reaction which is determinative of the presence of the protein in the presence of a heterogeneous population of proteins and other biologics. Thus, under designated conditions, a specified ligand binds preferentially to a particular protein and does not bind in a significant amount to other proteins present in the sample. A molecule or ligand (e.g., an antibody) that specifically binds to a protein has an association constant of at least 10^3 M^{-1} or 10^4 M^{-1}, sometimes 10^5 M^{-1} or 10^6 M^{-1}, in other instances 10^6 M^{-1} or 10^7 M^{-1}, preferably 10^8 M^{-1} to 10^9 M^{-1}, and more preferably, about 10^{10} M^{-1} to 10^{11} M^{-1} or higher. A variety of immunoassay formats can be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. *See*, *e.g.*, Harlow and Lane (1988) *Antibodies, A Laboratory Manual*, Cold Spring Harbor Publications, New York, for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity.

The term "isolated," "purified" or "substantially pure" means an object species (*e.g.*, a UCP-2 protein or nucleic acid) is the predominant macromolecular species present (*i.e.*, on a molar basis it is more abundant than any other individual species in the composition), and preferably the object species comprises at least about 50 percent (on a molar basis) of all macromolecular species present. Generally, an isolated, purified or substantially pure UCP-2 protein or nucleic acid will comprise more than 80 to 90 percent of all macromolecular species present in a composition. Most preferably, the object species is purified to essential homogeneity (*i.e.*, contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species.

The term "naturally occurring" as applied to an object means that the object can be found in nature.

The term "neurological disorder," "neurological injury," or "neuronal injury" generally refers to a disorder associated with some type of injury to neuronal cells or the death of neuronal death. Specific examples of such disorders include, but are not limited to, stroke, ischemic stroke, Parkinson's disease, Huntington's disease, inherited ataxias, motor

neuron diseases, Alzheimer's disease, epilepsy, and traumatic brain injury.

The term "apoptosis" has its general meaning in the art and refers to the process by which cells undergo a process of programmed cell death. Activation of caspase 3 is a marker for apoptosis. Apoptosis, including increased caspase 3 activity, has been implicated in various neurological disorders or neuronal injuries, e.g., stroke (Rosenblum, Stroke, 30: 1154-6; 1999; MacManus et al., J Neurotrauma, 17: 899-914, 2000; and Guglielmo et al., Neurol Res, 20: 283-96, 1998), traumatic brain injury (Raghupathi et al., J Neurotrauma, 17: 927-38, 2000), epilepsy (Timsit et al., Eur J Neurosci, 11: 263-78, 1999; and PitkAnen et al., Acta Neurol Scand Suppl, 162: 22-3, 1995), Parkinson's disease (Ziv et al., Mov Disord, 13: 865-70, 1998; and Tatton, Exp Neurol, 166: 29-43, 2000), and Alzheimer's disease (Hugon et al., J Neural Transm Suppl, 59: 125-31, 2000; and Masumura et al., Brain Res Mol Brain Res, 80: 219-27, 2000).

As used herein the term "stroke" has the meaning normally accepted in the art. The term can broadly refer to the development of neurological deficits associated with impaired blood flow regardless of cause. Potential causes include, but are not limited to, thrombosis, hemorrhage and embolism. The term "ischemic stroke" refers more specifically to a type of stroke that is of limited extent and caused due to blockage of blood flow.

A "tissue" refers to an aggregation of similar cells united in performance of a particular function. The tissue can be part of a living organism, a section excised from a living organism, or can be artificial. An artificial tissue is one in which the aggregation of cells are grown to function similar to a tissue in a living organism. The aggregated cells, however, are not obtained from a host (*i.e.*, a living organism). Artificial tissues can be grown *in vivo* or *in vitro*.

The term "detectably labeled" means that an agent (e.g., a probe) has been conjugated with a label that can be detected by physical, chemical, electromagnetic and other related analytical techniques. Examples of detectable labels that can be utilized include, but are not limited to, radioisotopes, fluorophores, chromophores, mass labels, electron dense particles, magnetic particles, spin labels, molecules that emit chemiluminescence, electrochemically active molecules, enzymes, cofactors, and enzyme substrates.

II. Overview

A variety of methods for diagnosing and treating individuals that have either suffered a neurological injury, that are at risk for neurological injury, or that have a neurological disorder are provided. High throughput screening methods to identify compounds effective in treating such individuals are also provided, as are compositions that include compounds identified through such screening methods. The methods and compositions find utility with a variety of neurological disorders, including stroke and, more specifically, ischemic stroke. The methods are based in part upon the finding that UCP-2 is differentially expressed (upregulated) in an ischemic preconditioning model in rat, indicating that UCP-2 exerts a protective effect against various neurological disorders, particularly stroke.

Certain methods are also based upon evidence indicating that UCP-2 inhibits certain components of an apoptotic cascade (see, e.g., Example 4). More specifically, the evidence indicates that UCP-2 may interfere with the effects of mitochondrial Ca^{2+} accumulation and the subsequent mitochondrial permeability transition (see, e.g., Example 4). Mitochondria have a large capacity for buffering Ca^{2+}, and during various toxic stimuli mitochondria accumulate large quantities of Ca^{2+}. However, excessive mitochondrial Ca^{2+} overload interfere with mitochondrial ATP production and lead to opening of the permeability transition pore (PTP). The PTP is a voltage-sensitive proteinaceous pore that allows solutes of <1,500 Daltons to equilibrate across the membrane (see D.G. Nicholls and S.L. Budd (2000) Physiological Reviews 80: 315-361). Opening of the pore results in dissipation of the mitochondrial membrane potential and mitochondrial swelling. Mitochondrial swelling induces release of cytochrome c into the cytosol where it interacts with apoptotic mediators. Pore formation can be inhibited, amongst other factors, by low matrix pH. UCP-2 is a mitochondrial proton transporter that leaks protons into the mitochondrial matrix. Thus, while not intending to be bound by any particular theory, it may be that activation of UCP-2 leads to a decreased matrix pH and subsequent prevention of PTP opening.

In view of the increase in UCP-2 expression observed in response to a neurological insult, the diagnostic and prognostic methods generally involve detecting the occurrence of a stroke or assessing an individual's susceptibility to stroke by detecting an elevated level of UCP-2 expression or activity in a sample obtained from the patient. Because of the protective effect provided by UCP-2, both therapeutic and prophylactic

treatment methods for individuals suffering or at risk of a neurological disorder such as stroke involve administering either a therapeutic or prophylactic amount of an agent that increases the activity of UCP-2. The agent that acts to increase UCP-2 activity can be a purified form of UCP-2, an agent that stimulates expression or synthesis of UCP-2, or a nucleic acid that includes a segment encoding UCP-2, or any agent that acts as an activator of the UCP-2 activity and function including but not limited to pharmacological agonists, or partial agonists. In view of the role of UCP-2 role as a potential regulator of mitochondrial permeability transition and release of cytochrome c, as well as an inhibitor of caspase-3 activation, the agent can also be one that has similar effects on PTP, cytochrome c release and caspase-3 activation.

The screening methods generally involve conducting various types of assays to identify agents that upregulate the expression or activity of UCP-2. Such screening methods can initially involve screens to identify compounds that can bind to UCP-2. Certain assays are designed to measure more clearly the effect that different agents have on UCP-2 activities or expression levels. Other screening methods are designed to identify compounds that influence mitochondrial permeability transition and inhibit caspase-3 activation as does UCP-2. Lead compounds identified during these screens can serve as the basis for the synthesis of more active analogs. Lead compounds and/or active analogs generated therefrom can be formulated into pharmaceutical compositions effective in treating neurological disorders such as stroke.

III. Differential Expression of UCP-2

The mammalian brain has a limited capacity to survive long periods of hypoxia and ischemia (lack of oxygen and blood supply). Following exposure to hypoxia-ischemia, neurons die by rapid or slow mechanisms of cell death (necrosis or apoptosis). Hypoxic-ischemic brain insults, such as stroke, neonatal asphyxia, heart failure (prolonged lack of blood supply to the brain), or drowning, can cause severe permanent brain damage.

On the other hand, brief, sublethal periods of hypoxia-ischemia can lead to a transient phase in the brain when neurons become protected from subsequent injury and death. This treatment, generally referred to as ischemic tolerance, or ischemic preconditioning, can provide the basis for and lead to an understanding of intrinsic protective mechanisms and pathways through endogenous proteins or factors that provide for this effect.

Thus, as used herein, ischemic preconditioning refers to a brief, transient, non-destructive stroke that triggers intrinsic neuroprotective mechanisms.

Stroke can be modeled in animals, such as the rat, by occluding certain cerebral arteries that prevent blood from flowing into particular regions of the brain, then releasing the occlusion and permitting blood to flow back into that region of the brain (reperfusion). These focal ischemia models are different than global ischemia models where blood flow to the entire brain is blocked for a period of time prior to reperfusion. Certain regions of the brain are particularly sensitive to this type of global ischemic insult. The hippocampus, and more specifically the CA1 region of the hippocampus, is primarily affected by global ischemia. Ten minutes of global ischemia induce profound selective neuronal loss in the CA1 region of hippocampus with non-detectable neuronal damage in CA3 region and dentate gyrus of hippocampus. With increasing periods of global ischemia, delayed cell death can also be detected in the striatum and layers 2 and 5 of the cerebral cortex (Lipton (1999) Physiol. Rev. 79: 1431-1568). In contrast, in focal ischemia, the precise region that is directly damaged is dictated by the location of the blockade and duration of ischemia prior to reperfusion. In animal models of focal ischemia there is, like in the human condition, a gradation of ischemia from the infarct core of the lesion to the outermost boundary, and hence there are different metabolic conditions within the affected site. Because of its duration and heterogeneity, the insult is complex.

In the rat, ten minutes global ischemia (Two-Vessel occlusion model with hypotension, Lipton (1999) Physiol. Rev. 79: 1431-1568) is sufficient to induce the complete destruction of CA1 neurons in the hippocampus. However, a three-minute ischemic event and several hours of recovery time are sufficient to effectively reduce the damage of a ten-minute ischemic insult. This neuroprotective effect is dependent on de novo protein synthesis. Therefore, genes that are specifically upregulated in an ischemic preconditioning model may be neuroprotective, either directly or indirectly, whereas longer ischemic times may lead to the induction of other genes that have neuro-damaging properties. The rat model of both ischemic preconditioning and global ischemia is highly relevant because it duplicates the ischemia/reperfusion that occurs in the human brain during drowning, cardiac by-pass surgery and cardiac arrest.

As described in greater detail in Example 1, a number of genes that are induced in the hippocampus by such protective hypoxic-ischemic treatment have been

identified using rat model systems. These genes were identified by performing differential cloning between preconditioned and normal rat brains and sequencing the differentially expressed genes. This sequence information was subsequently utilized to conduct sequence comparisons with sequences available in public databases using standard sequence algorithms (e.g., BLAST). Of the differentially expressed genes identified, four independent clones were identified that match the sequence for rat UCP-2 (Genbank ID: AB010743). UCP-2 upregulation in an ischemic preconditioning model in which preconditioning confers a protective effect against subsequent neurological insults indicates that an increase in UCP-2 activity can have a neuroprotective effect on various neurological cell types (e.g., neurons, glial cells, microglial cells), thereby protecting against various neurological disorders, including but not limited to, stroke and ischemic stroke. Thus, agents able to increase the expression levels or activity of UCP-2 can potentially have a neuroprotective effect.

The role that UCP-2 plays in regulating mitochondrial membrane potential also indicates that agents that alter mitochondrial permeability are also candidates for providing a neuroprotective effect. As described more fully in the Examples below, the evidence indicates that the mechanism of action for UCP-2 involves inhibition of cell apoptosis, which in turn is a consequence of a cascade of events involving the mitochondrial permeability transition.

Apoptosis, or programmed cell death, plays a fundamental role in normal biological processes as well as in several disease states (see, e.g., Nicholson and Thornberry, (1996) Trends Biochem. Sci. 22:299-306; and Thompson (1995) Science 281:1312-1316). Apoptosis can be induced by various stimuli that all produce the same end result: systematic and deliberate cell death. One apoptotic cascade is triggered by mitochondrial permeability transition which consists in the opening of a voltage-sensitive pore that allows solutes to equilibrate across the mitochondrial membrane. Mitochondria participate in apoptotic signaling by mediating the activation of caspases via release of cytochrome c to the cytosol. Thus, localization of cytochrome c serves as a convenient marker for studying mitochondrial involvement in apoptosis. Caspases are cysteine proteases that possess the unusual ability to cleave substrates after aspartate residues; this activity is central to their role in apoptosis. Upon activation, caspases disable cellular homeostatic and repair processes, and cleave important structural components in the cell. Caspase-3 plays a direct role in proteolytic

digestion of cellular proteins responsible for progression to apoptosis (see, e.g., Fernandes-Alnemri et al. (1994) J. Biol. Chem. 269:30761-30764).

The mechanisms underlying the neuroprotective role of UCP-2 may include inhibition and/or regulation of any of the components of the apoptotic cascade including, but not limited to, effects on mitochondrial membrane potential and mitochondrial permeability transition, blockade of cytochrome c release from mitochondria, and activation of caspases, as evidenced in the Examples below. That UCP-2 is a mitochondrial protein capable of lowering the mitochondrial matrix pH, reducing free-radical levels and ATP production, which are involved in neuronal apoptosis, is consistent with such a mechanism.

The finding of UCP-2 upregulation as a mechanism for providing a protective neurological effect and the evidence indicating its role in inhibiting apoptosis provides the basis for a number of diagnostic and therapeutic methods, as well as screening methods. Agents that influence UCP-2 activity or expression can potentially provide a more effective neuroprotective effect than agents that interact with a downstream component of an apoptotic cascade (e.g., caspase 3). Because UCP-2 occurs early in the apoptotic cascade, it has the potential to affect a greater number of cellular pathways than a component that is further downstream in the cascade. These various diagnostic, treatment and screening methods are discussed further below.

IV. Diagnostic and Prognostic Methods

The differential expression of UCP-2 in response to an ischemic event indicates that UCP-2 can serve as a marker for diagnosing individuals that have suffered a mild stroke, and in prognostic evaluations to detect individuals at risk for stroke. Prognostic methods can also be utilized in the assessment of the severity of the stroke and the likelihood of recovery.

In general, such diagnostic and prognostic methods involve detecting an elevated level of UCP-2 in the cells or tissue of an individual or a sample therefrom. A variety of different assays can be utilized to detect an increase in UCP-2 expression, including both methods that detect UCP-2 transcript and UCP-2 protein levels. More specifically, the diagnostic and prognostic methods disclosed herein involve obtaining a sample from an individual and determining at least qualitatively, and preferably quantitatively, the level of UCP-2 expression in the sample. Usually this determined value or

test value is compared against some type of reference or baseline value. Details regarding samples, methods for quantitating expression levels and controls are set forth in the following sections.

A. Detection of Transcript

A number of different methods for detecting and optionally quantitating UCP-2 transcript are available and known to those of skill in the art. Examples of suitable methods for detecting an quantifying changes in UCP-2 expression include, but are not limited to, dot blots, in-situ hybridization, nucleic acid probe arrays, quantitative reverse-transcription PCR, (RT-PCR), Northern blots and RNAase protection methods.

1. Dot Blots and In-situ Hybridization

Dot blots are one example of an assay that can be utilized to determine the amount of UCP-2 transcript present in a nucleic acid sample obtained from an individual being tested. In these assays, a sample from an individual being tested for stroke is spotted on a support (e.g., a filter) and then probed with labeled nucleic acid probes that specifically hybridize with UCP-2 nucleic acids. After the probes have been allowed to hybridize to the immobilized nucleic acids on the filter, unbound nucleic acids are rinsed away and the presence of hybridization complexes detected and quantitated on the basis of the amount of labeled probe bound to the filter.

In-situ hybridization methods are hybridization methods in which the cells are not lysed prior to hybridization. Because the method is performed in situ, it has the advantage that it is not necessary to prepare RNA from the cells. The method usually involves initially fixing test cells to a support (e.g., the walls of a microtiter well) and then permeabilizing the cells with an appropriate permeabilizing solution. A solution containing labeled probes for UCP-2 is then contacted with the cells and the probes allowed to hybridize with UCP-2 nucleic acids. Excess probe is digested, washed away and the amount of hybridized probe measured. This approach is described in greater detail by Harris, D. W. (1996) Anal. Biochem. 243:249-256; Singer, et al. (1986) Biotechniques 4:230-250; Haase et al. (1984) Methods in Virology, vol. VII, pp. 189-226; and Nucleic Acid Hybridization: A Practical Approach (Hames, et al., eds., 1987).

The hybridization probes utilized in the foregoing methods are polynucleotides that are of sufficient length to specifically hybridize to a UCP-2 nucleic acid. Hybridization probes are typically at least 15 nucleotides in length, in some instances 20 to 30 nucleotides in length, in other instances 30 to 50 nucleotides in length, and in still other instances up to the full length of a UCP-2 nucleic acid. The probes are labeled with a detectable label, such as a radiolabel, fluorophore, chromophore or enzyme to facilitate detection. Methods for synthesizing the necessary probes include the phosphotriester method described by Narang et al. (1979) Methods of Enzymology 68:90, and the phosphodiester method disclosed by Brown et al. (1979) Methods of Enzymology 68:109.

2. Nucleic Acid Probe Arrays

Related hybridization methods utilize nucleic acid probe arrays to detect and quantitate UCP-2 transcript. The arrays utilized to detect UCP-2 can be of varying types. The probes utilized in the arrays can be of varying types and can include, for example, synthesized probes of relatively short length (e.g., a 20-mer or a 25-mer), cDNA (full length or fragments of gene), amplified DNA, fragments of DNA (generated by restriction enzymes, for example) and reverse transcribed DNA (see, e.g., Southern et al. (1999) Nature Genetics Supplement 21:5-9 (1999). Both custom and generic arrays can be utilized in detecting UCP-2 expression levels. Custom arrays can be prepared using probes that hybridize to particular preselected subsequences of mRNA gene sequences of UCP-2 or amplification products prepared from them. Generic arrays are not specially prepared to bind to UCP-2 sequences but instead are designed to analyze mRNAs irrespective of sequence. Nonetheless, such arrays can still be utilized because UCP-2 nucleic acids only hybridize to those locations that include complementary probes. Thus, UCP-2 levels can still be determined based upon the extent of binding at those locations bearing probes of complementary sequence.

In probe array methods, once nucleic acids have been obtained from a test sample, they typically are reversed transcribed into labeled cDNA, although labeled mRNA can be used directly. The test sample containing the labeled nucleic acids is then contacted with the probes of the array. After allowing a period sufficient for any labeled UCP-2 nucleic acid present in the sample to hybridize to the probes, the array is typically subjected to one or more high stringency washes to remove unbound nucleic acids and to minimize nonspecific

binding to the nucleic acid probes of the arrays. Binding of labeled UCP-2 is detected using any of a variety of commercially available scanners and accompanying software programs.

For example, if the nucleic acids from the sample are labeled with fluorescent labels, hybridization intensity can be determined by, for example, a scanning confocal microscope in photon counting mode. Appropriate scanning devices are described by *e.g.*, U.S. 5,578,832 to Trulson *et al.*, and U.S. 5,631,734 to Stern *et al.* and are available from Affymetrix, Inc., under the GeneChip™ label. Some types of label provide a signal that can be amplified by enzymatic methods (*see* Broude, *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 91, 3072-3076 (1994)). A variety of other labels are also suitable including, for example, radioisotopes, chromophores, magnetic particles and electron dense particles.

Those locations on the probe array that are hybridized to labeled nucleic acid are detected using a reader, such as described by U.S. Patent No. 5,143,854, WO 90/15070, and U.S. 5,578,832. For customized arrays, the hybridization pattern can then be analyzed to determine the presence and/or relative amounts or absolute amounts of known mRNA species in samples being analyzed as described in *e.g.*, WO 97/10365.

Further guidance regarding the use of probe arrays sufficient to guide one of skill in the art is provided in WO 97/10365, PCT/US/96/143839 and WO 97/27317. Additional discussion regarding the use of microarrays in expression analysis can be found, for example, in Duggan, et al., Nature Genetics Supplement 21:10-14 (1999); Bowtell, Nature Genetics Supplement 21:25-32 (1999); Brown and Botstein, Nature Genetics Supplement 21:33-37 (1999); Cole et al., Nature Genetics Supplement 21:38-41 (1999); Debouck and Goodfellow, Nature Genetics Supplement 21:48-50 (1999); Bassett, Jr., et al., Nature Genetics Supplement 21:51-55 (1999); and Chakravarti, Nature Genetics Supplement 21:56-60 (1999).

3. Quantitative RT-PCR

A variety of so-called "real time amplification" methods or "real time quantitative PCR" methods can also be utilized to determine the quantity of UCP-2 mRNA present in a sample. Such methods involve measuring the amount of amplification product formed during an amplification process. Fluorogenic nuclease assays are one specific example of a real time quantitation method that can be used to detect and quantitate UCP-2 transcript. In general such assays continuously measure PCR product accumulation using a

dual-labeled fluorogenic oligonucleotide probe -- an approach frequently referred to in the literature simply as the "TaqMan" method.

The probe used in such assays is typically a short (ca. 20-25 bases) polynucleotide that is labeled with two different fluorescent dyes. The 5' terminus of the probe is typically attached to a reporter dye and the 3' terminus is attached to a quenching dye, although the dyes can be attached at other locations on the probe as well. For measuring UCP-2 transcript, the probe is designed to have at least substantial sequence complementarity with a probe binding site on UCP-2 transcript. Upstream and downstream PCR primers that bind to regions that flank UCP-2 are also added to the reaction mixture for use in amplifying UCP-2.

When the probe is intact, energy transfer between the two fluorophors occurs and the quencher quenches emission from the reporter. During the extension phase of PCR, the probe is cleaved by the 5' nuclease activity of a nucleic acid polymerase such as Taq polymerase, thereby releasing the reporter dye from the polynucleotide-quencher complex and resulting in an increase of reporter emission intensity that can be measured by an appropriate detection system.

One detector which is specifically adapted for measuring fluorescence emissions such as those created during a fluorogenic assay is the ABI 7700 manufactured by Applied Biosystems, Inc. in Foster City, CA. Computer software provided with the instrument is capable of recording the fluorescence intensity of reporter and quencher over the course of the amplification. These recorded values can then be used to calculate the increase in normalized reporter emission intensity on a continuous basis and ultimately quantify the amount of the mRNA being amplified.

Additional details regarding the theory and operation of fluorogenic methods for making real time determinations of the concentration of amplification products are described, for example, in U.S. Pat Nos. 5,210,015 to Gelfand, 5,538,848 to Livak, et al., and 5,863,736 to Haaland, as well as Heid, C.A., et al., Genome Research, 6:986-994 (1996); Gibson, U.E.M, et al., Genome Research 6:995-1001 (1996); Holland, P. M., et al., Proc. Natl. Acad. Sci. USA 88:7276-7280, (1991); and Livak, K.J., et al., PCR Methods and Applications 357-362 (1995), each of which is incorporated by reference in its entirety.

4. Northern Blots

Northern blots can be used to detect and quantitate UCP-2 transcript. Such methods typically involve initially isolating total cellular or poly(A) RNA and separating the RNA on an agarose gel by electrophoresis. The gel is then overlaid with a sheet of nitrocellulose, activated cellulose, or glass or nylon membranes and the separated RNA transferred to the sheet or membrane by passing buffer through the gel and onto the sheet or membrane. The presence and amount of UCP-2 transcript present on the sheet or membrane can then be determined by probing with a labeled probe complementary to UCP-2 to form labeled hybridization complexes that can be detected and optionally quantitated (see, e.g., . Sambrook, et al. (1989) Molecular Cloning – A Laboratory Manual (2nd ed) Vols. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, NY).

5. <u>RNAase Protection Assays</u>

Ribonuclease protection assays (RPA) involve preparing a labeled antisense RNA probe for UCP-2. This probe is subsequently allowed to hybridize in solution with UCP-2 transcript contained in a biological sample to form RNA:RNA hybrids. Unhybridized RNA is then removed by digestion with an RNAase, while the RNA:RNA hybrid is protected from degradation. The labeled RNA:RNA hybrid is separated by gel electrophoresis and the band corresponding to UCP-2 detected and quantitated. Usually the labeled RNA probe is radiolabeled and the UCP-2 band detected and quantitated by autoradiography. RPA is discussed further by (Lynn et al. (1983) Proc. Natl. Acad. Sci. 80:2656; Zinn, et al. (1983) Cell 34:865; and Sambrook, et al. (1989) Molecular Cloning – A Laboratory Manual (2nd ed) Vols. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, NY).

B. <u>Detection of UCP-2 Translation Product</u>

Instead of detecting an increase in transcript, another option for detecting UCP-2 expression is to determine UCP-2 protein levels and/or activity. A number of different approaches can be utilized to accomplish this, including the use of antibodies that specifically bind UCP-2 and assays that measure UCP-2 activity (e.g., mitochondrial respiration).

1. <u>Immunological Methods</u>

One method for determining the expression level of UCP-2 is to utilize antibodies that specifically bind to UCP-2 to capture UCP-2 from a sample. One such approach is the so-called "sandwich immunoassay." Such methods generally involve contacting a sample from an individual with immobilized anti-UCP-2 antibodies which capture UCP-2 from the sample to form a complex. This complex is subsequently contacted with a labeled anti-UCP-2 detection antibody that preferably recognizes a different portion of UCP-2 then the immobilized antibody. This detection antibody binds to the complex containing UCP-2 and immobilized antibody to form a ternary complex that can be quantitated based upon the magnitude of a signal generated by the labeled detection antibody. Certain of the sandwich assays are enzyme-linked immunosorbent assays (ELISA) in which the detection antibody bears an enzyme. The detection antibody is detected by providing a substrate for the enzyme to generate a detectable signal.

Further guidance regarding the methodology and steps of a variety of antibody assays is provided, for example, in U.S. Patent No. 4,376,110 to Greene; "Immunometric Assays Using Monoclonal Antibodies," in *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Chap. 14 (1988); Bolton and Hunter, "Radioimmunoassay and Related Methods," in *Handbook of Experimental Immunology* (D.M. Weir, ed.), Vol. 1, chap. 26, Blackwell Scientific Publications, 1986; Nakamura, et al., "Enzyme Immunoassays: Heterogeneous and Homogenous Systems," in *Handbook of Experimental Immunology* (D.M. Weir, ed.), Vol. 1, chap. 27, Blackwell Scientific Publications, 1986; and Current Protocols in Immunology, (John E. Coligan, et al., eds), chap. 2, section I, (1991).

The antibodies used to perform the foregoing assays can include polyclonal antibodies, monoclonal antibodies and fragments thereof as described supra. Monoclonal antibodies can be prepared according to established methods (see, e.g., Kohler and Milstein (1975) Nature 256:495; and Harlow and Lane (1988) Antibodies: A Laboratory Manual (C.H.S.P., N.Y.)).

2. <u>Activity Assays</u>

Various different UCP-2 activities can also be determined to detect an increase in UCP-2 expression. For example, given its uncoupling role in mitochondria, certain assays involve detecting an increase in mitochondrial respiration mediated by UCP-2 in a sample from a patient potentially suffering from stroke or at risk for stroke relative to a baseline

value. Assays can be conducted using isolated cells or tissue samples, or isolated mitochondrial preparations. Instead of measuring mitochondrial respiration, one can instead measure the extent of mitochondrial swelling. Methods for conducting such mitochondrial assays are known in the art and described, for example, by Salvioli et al. (1997) FEBS Lett 411:77-82; and Smiley et al. (1991) Proc. Natl. Acad. Sci. USA 88:3671-3675). Methods for conducting such assays with certain uncoupling proteins is discussed, for example, in PCT publications WO 00/17353 and WO 98/45313.

By analogy to UCP-1 activity, another activity that can serve as a measure of UCP-2 activity in some instances is the transport of fatty acids by UCP-2. UCP-1 proton transport activity is regulated by fatty acids. In vitro studies also show that UCP-1 can function as a fatty acid anion transporter. It is believed that fatty acids stimulate proton transport across the mitochondrial membrane by themselves mediating the transport of protons as UCP-1 protonophores (see, e.g., Garlid, K.D., et al. (1996) J. Biol. Chem. 271:2615-2620). The sequence homology between UCP-1 and UCP-2 indicates that UCP-2 activity also includes fatty acid transport. Such assays can be conducted using labeled (e.g., radiolabeled) fatty acids.

C. Time Course Analyses

Certain prognostic methods of assessing a patient's risk of stroke involve monitoring UCP-2 expression levels for a patient susceptible to stroke to track whether there appears to be an increase in UCP-2 expression over time. An increase in UCP-2 expression over time can indicate that the individual is at increased risk for stroke. As with other measures of UCP-2, the UCP-2 expression level for the patient at risk for stroke is compared against a baseline value (see infra). The baseline in such analyses can be a prior value determined for the same individual or a statistical value (e.g., mean or average) determined for a control group (e.g., a population of individuals with no apparent neurological risk factors). An individual showing a statistically significant increase in UCP-2 expression levels over time can prompt the individual's physician to take prophylactic measures to lessen the individual's potential for stroke. For example, the physician can recommend certain life style changes (e.g., improved diet, exercise program) to reduce the risk of stroke. Alternatively, or in addition, the physician can prescribe medicines to reduce the stroke risk.

D. Controls

The various test values determined for a sample from an individual believed to have suffered a stroke or to be susceptible to stroke typically are compared against a baseline value to assess the extent of increased UCP-2 expression, if any. This baseline value can be any of a number of different values. In some instances, the baseline value is a value established in a trial using a healthy cell or tissue sample that is run in parallel with the test sample. Alternatively, the baseline value can be a statistical value (e.g., a mean or average) established from a population of control cells or individuals. For example, the baseline value can be a value or range which is characteristic of a control individual or control population. For instance, the baseline value can be a statistical value or range that is reflective of UCP-2 levels for the general population, or more specifically, healthy individuals not susceptible to stroke. Individuals not susceptible to stroke generally refer to those having no apparent risk factors correlated with stroke, such as high blood pressure, high cholesterol levels, diabetes, smoking and high salt diet, for example.

E. Samples

Samples can be obtained from a variety of sources. For example, since the methods are designed primarily to diagnosis and assess risk factors for humans to neurological disorders such as stroke, samples are typically obtained from a human subject. However, the methods can also be utilized with samples obtained from all other mammals, such as non-human primates (e.g., apes and chimpanzees), mice and rats. Such samples can be referred to as a patient sample or a biological sample.

Samples can be obtained from the tissues or fluids of an individual, as well as from cell cultures or tissue homogenates. For example, samples can be obtained from whole blood, serum, semen, saliva, tears, urine, fecal material, sweat, buccal, skin, spinal fluid and amniotic fluid. Samples can also be derived from *in vitro* cell cultures, including the growth medium, recombinant cells and cell components.

Because certain diagnostic methods involve evaluating the level of expression in nerve cells, the sample can be obtained from various types of nerve cells including, but not limited to, neuron cells, glial cells, microglial cells and cortical neuron cells. Current evidence indicates that one consequence of stroke is that the blood/brain barrier becomes more permeable. Stroke also results in the death of certain cells which, upon dying, are

lysed, thus expelling cellular components such as UCP-2. These components can then traverse the blood/brain barrier and be picked up by the circulatory system. Consequently, certain diagnostic and prognostic methods are conducted with blood samples.

Because UCP-2 is expressed as part of a neuroprotective response, diagnostic samples are collected any time after an individual is suspected to have had a stroke or to exhibit symptoms that are predictors of stroke. In prophylactic testing, samples can be obtained from an individual who present with risk factors that indicate a susceptibility to stroke (e.g., high blood pressure, obesity, diabetes) as part of a routine assessment of the individual's health status.

Some of the diagnostic and prognostic methods that involve the detection of UCP-2 transcript begin with the lysis of cells and subsequent purification of nucleic acids from other cellular material. To measure the transcription level (and thereby the expression level) of UCP-2, a nucleic acid sample comprising mRNA transcript(s) of UCP-2, or nucleic acids derived from the mRNA transcript(s) is obtained. A nucleic acid derived from an mRNA transcript refers to a nucleic acid for whose synthesis the mRNA transcript, or a subsequence thereof, has ultimately served as a template. Thus, a cDNA reverse transcribed from an mRNA, an RNA transcribed from that cDNA, a DNA amplified from the cDNA, an RNA transcribed from the amplified DNA, are all derived from the mRNA transcript and detection of such derived products is indicative of the presence and/or abundance of the original transcript in a sample. Thus, suitable samples include, but are not limited to, mRNA transcripts of UCP-2, cDNA reverse transcribed from the mRNA, cRNA transcribed from the cDNA, DNA amplified from UCP-2 nucleic acids, and RNA transcribed from amplified DNA.

In some methods, a nucleic acid sample is the total mRNA isolated from a biological sample; in other instances, the nucleic acid sample is the total RNA from a sample taken from an individual. Any RNA isolation technique such as those described supra that do not select against the isolation of mRNA can be utilized for the purification of such RNA samples. If needed to improve the detection limits of the method, UCP-2 can be amplified prior to further analysis using established amplification techniques such as those described above.

IV. Therapeutic/Prophylactic Treatment Methods

A. General

The upregulation of UCP-2 detected in the neuroprotection model system indicates that methods that increase the expression or activity of UCP-2 can be utilized in treating individuals that have suffered a neuronal injury, as well as prophylactically treating individuals at risk for neuronal injury. In general, such methods involve administering to an individual that has suffered a neurological injury or that is at risk for such injury, an agent in an amount effective to increase the expression or activity of UCP-2. The neurological injury being treated can include, stroke (particularly ischemic stroke), and all other neurological diseases associated with altered mitochondrial function including, but not limited to, Alzheimer's Disease, Parkinson's Disease, Huntington's Disease, inherited ataxias, schizophrenia, dementia, mitochondrial encephalopathy, amylotrophic lateral sclerosis, motor neuron diseases and others (see, e.g., Beal (2000) TINS 23: 298). In a broader view, mitochondrial dysfunction is a critical factor in cell death by necrosis and apoptosis. Thus, many diseases (neurological and peripheral) involving cell death by apoptosis and/or necrosis can be targeted by an increase in UCP-2 activity (e.g., myocardial ischemia, diabetes, hepatic cierrosis, muscular dystrophies, spinal cord injuries).

Therapeutic/prophylactic intervention to increase UCP-2 expression and/or activity include but are not limited to administration of UCP-2 inducers shortly after an ischemic episode, and chronic administration in individuals with a previous stroke, at higher risk for stroke, and in genetically predisposed individuals.

Depending upon the individual's condition, the agent can be administered in a therapeutic or prophylactic amount. If the individual has suffered a neurological injury, then, at least for some period of time after the injury, the agent is typically administered in a therapeutic amount. A "therapeutic amount," as defined herein, means an amount sufficient to remedy a neurological disease state or symptoms, or otherwise prevent, hinder, retard or reverse the progression of a neurological disease or any other undesirable symptoms, especially stroke and more particularly ischemic stroke. If, however, the individual only presents with risk factors suggesting he or she is susceptible to neurological injury, then the agent is administered in a prophylactically effective amount. A prophylactic amount can also be administered as part of a long-term regimen for individuals that have already had a stroke and are at increased risk of another stroke. A "prophylactic amount" is an amount sufficient

to prevent, hinder or retard a neurological disease or any undesirable symptom, particularly with regard to neurological disorders such as stroke, particularly ischemic stroke.

Prophylactic treatment can commence whenever an individual is at increased risk of suffering from a neurological disorder such as stroke. For example, individuals having risk factors known to be correlated with stroke can be administered prophylactic amounts of an agent that increases UCP-2 activity. Examples of such individuals include those that: are overweight or obese, have high blood pressure, have elevated cholesterol levels, have diabetes and/or are about to undergo medial treatment that puts the individual at risk (e.g., a patient about to undergo cardiac by-pass surgery).

In view of UCP-2 activity as a mitochondrial protein that regulates mitochondrial permeability transition, agents utilized in therapeutic methods can include those that affect the mitochondrial transition pore in a similar manner. Similarly, given the evidence indicating that UCP-2 inhibits apoptosis at least in part by inhibiting caspase 3 activation, therapeutic agents can also include agents with similar inhibitory properties.

B. UCP-2 and Other Agents

A variety of different agents can be administered to achieve the desired increase in UCP-2 activity. In some instances, the agent is a purified UCP-2 polypeptide as defined supra, including active fragments thereof. Methods for preparing purified UCP-2 are described infra. Other therapeutic agents that are administered act to stimulate the synthesis or expression of UCP-2. Such agents include those that induce the UCP-2 promoter, for example, thereby increasing expression of UCP-2 in cells. Compounds having such activity can be identified using the screening methods described below in the screening section. Often such compounds are administered in combination with a pharmaceutically-acceptable carrier. Such carriers and modes of administration are discussed further in the section on pharmaceutical compositions infra. Various inducers of UCP-2 can be utilized in certain methods. Specific examples of such inducers include PPARγ agonists such as β3-agonists such as isoproterenol. Inducers can also include agents that activate the transcription of UCP-2.

Compounds increasing UCP-2 activity can be administered in combination with various other compounds. For example, the compound can be administered with an agent that increases the permeability of the blood/brain barrier to facilitate delivery of the

agent activating UCP-2 activity to the brain. Such agents include, but are not limited to, bradykinin, serotonin, histamine and arachidonic acid. Other compounds that can be administered with the compound increasing UCP-2 activity include compounds that protect against clotting and prevent thrombus formation including but not limited to heparin and fucoidan.

Because UCP-2 is transmembrane protein of the inner mitochondrial membrane that functions as a proton channel (Ricquier and Bouillaud (2000) Biochem J 345: 161-179), identification of agonists that increase UCP-2 activity are another therapeutic option (see, generally, Drews (2000) Science 287: 1960 for a discussion of drug targets to ion channels). These agonists can gate the UCP-2 channel in the absence of physiological regulation and gating mechanisms and lead to an increase in UCP-2-mediated proton flow.

Additional agents that can be administered in combination with the identified compounds and delivery mode options are discussed in detail in the pharmaceutical composition section infra.

C. Gene Therapy

Gene therapy is another option for increasing UCP-2 expression. Such methods generally involve administering to an individual a nucleic acid molecule that encodes UCP-2 or an active fragment thereof. The administered nucleic acid increases the level of UCP-2 expression in one or more tissues, especially nerve cells, and particularly neuron cells. The nucleic acid is administered to achieve synthesis of UCP-2 in an amount effective to obtain a therapeutic or prophylactic effect in the individual receiving the therapy. As used herein, the term "gene therapy" refers to therapies in which a lasting effect is obtained with a single treatment, and methods wherein the gene therapeutic agents are administered multiple times to achieve or maintain the desired increase in UCP-2 expression.

The nucleic acid molecules encoding UCP-2 can be administered ex vivo or in vivo. Ex vivo gene therapy methods involve administering the nucleic acid to cells in vitro and then transplanting the cells containing the introduced nucleic acid back into the individual being treated. Techniques suitable for the in vitro transfer of UCP-2 nucleic acids into mammalian cells include, but are not limited to, the use of liposomes, electroporation, microinjection, cell fusion, DEAE-dextran and calcium phosphate precipitation methods. Once the cells have been transfected, they are subsequently introduced into the patient.

Certain ex vivo methods are based on the use of any form of genetically-modified neuronal stem cells for the continuous intracerebral delivery of UCP-2. For example, UCP-2 producing cells can be implanted or surrounded by a semipermeable membrane (e.g., a capsule), directly into the intracerebroventricular space or into the cerebrospinal fluid.

In vivo gene therapy methods involve the direct administration of nucleic acid or a nucleic acid/protein complex into the individual being treated. In vivo administration can be accomplished according to a number of established techniques including, but not limited to, injection of naked nucleic acid, viral infection, transport via liposomes and transport by endocytosis. Of these, transfection with viral vectors and viral coat protein-liposome mediated transfection are commonly used methods (see, e.g., Dzau et al (1993) Trends in Biotechnology 11:205-210). Suitable viral vectors include, for example, adenovirus, adeno-associated virus and retrovirus vectors.

Methods can be designed to selectively deliver nucleic acids to certain cells. Examples of such cells include, neurons, astrocytes, oligodendrocytes, microglia, and endothelial cells. Because UCP-2 exhibits a neuroprotective effect, certain treatment methods are designed to selectively express UCP-2 in neuron cells and/or target the nucleic acid for delivery to nerve cells. However, in other instances non-nerve cells are targeted (see, e.g., microglia, astrocytes, endothelial cells, oligodendrocytes). One technique for achieving selective expression in nerve cells is to operably link the nucleic acid encoding UCP-2 to a promoter that is primarily active in nerve cells. Examples of such promoters include, but are not limited to, prion protein promoter, calcium-calmodulin dependent protein kinase promoter, enolase promoter and PDGFβ-promoter. Alternatively, or in addition, the nucleic acid can be administered with an agent that targets the nucleic acid to nerve cells. For instance, the nucleic acid can be administered with an antibody that specifically binds to a cell-surface antigen on the nerve cells or a ligand for a receptor on neuronal cells. When liposomes are utilized, substrates that bind to a cell-surface membrane protein associated with endocytosis can be attached to the liposome to target the liposome to nerve cells and to facilitate uptake. Examples of proteins that can be attached include capsid proteins or fragments thereof that bind to nerve cells, antibodies that specifically bind to cell-surface proteins on nerve cells that undergo internalization in cycling and proteins that target intracellular localizations within nerve cells (see, e.g., Wu et al. (1987) J. Biol. Chem.

262:4429-4432; and Wagner, et al. (1990) Proc. Natl. Acad. Sci. USA 87:3410-3414). Gene marking and gene therapy protocols are reviewed by Anderson et al. (1992) Science 256:808-813.

Various other delivery options can also be utilized. For instance, a nucleic acid containing UCP-2 (e.g., a vector containing UCP-2) can be injected directly into the cerebrospinal fluid. Alternatively, such nucleic acids can be administered by intraventricular injections.

V. Screening Methods

A number of different screening protocols can be utilized to identify agents that increase the level of expression of UCP-2 in cells, particularly mammalian cells, especially human cells. In general terms, the screening methods involve screening a plurality of agents to identify an agent that increases the activity of UCP-2 by binding to UCP-2, preventing an inhibitor from binding to UCP-2 or activating expression of UCP-2, for example.

A. UCP-2 Binding Assays

Preliminary screens can be conducted by screening for compounds capable of binding to UCP-2, as at least some of the compounds so identified are likely UCP-2 activators. The binding assays usually involve contacting a UCP-2 protein with one or more test compounds and allowing sufficient time for the protein and test compounds to form a binding complex.. Any binding complexes formed can be detected using any of a number of established analytical techniques. Protein binding assays include, but are not limited to, methods that measure co-precipitation, co-migration on non-denaturing SDS-polyacrylamide gels, and co-migration on Western blots (see, e.g., Bennet, J.P. and Yamamura, H.I. (1985) "Neurotransmitter, Hormone or Drug Receptor Binding Methods," in *Neurotransmitter Receptor Binding* (Yamamura, H. I., et al., eds.), pp. 61-89. The UCP-2 protein utilized in such assays can be naturally expressed, cloned or synthesized UCP-2.

B. Expression Assays

Certain screening methods involve screening for a compound that up-regulates the expression of UCP-2. Such methods generally involve conducting cell-based assays in

which test compounds are contacted with one or more cells expressing UCP-2 and then detecting and an increase in UCP-2 expression (either transcript or translation product). Some assays are performed with neuron cells that express endogenous UCP-2 (e.g., cortical neuron cells, glial cells or microglial cells). Other expression assays are conducted with non-neuronal cells that express an exogenous UCP-2.

UCP-2 expression can be detected in a number of different ways. As described infra, the expression level of UCP-2 in a cell can be determined by probing the mRNA expressed in a cell with a probe that specifically hybridizes with a transcript (or complementary nucleic acid derived therefrom) of UCP-2. Probing can be conducted by lysing the cells and conducting Northern blots or without lysing the cells using in situ-hybridization techniques (see above). Alternatively, UCP-2 protein can be detected using immunological methods in which a cell lysate is probe with antibodies that specifically bind to UCP-2.

Other cell-based assays are reporter assays conducted with cells that do not express UCP-2. Certain of these assays are conducted with a heterologous nucleic acid construct that includes a UCP-2 promoter that is operably linked to a reporter gene that encodes a detectable product. Suitable UCP-2 promoters are described, for example, in PCT Publication WO 00039315. A number of different reporter genes can be utilized. Some reporters are inherently detectable. An example of such a reporter is green fluorescent protein that emits fluorescence that can be detected with a fluorescence detector. Other reporters generate a detectable product. Often such reporters are enzymes. Exemplary enzyme reporters include, but are not limited to, β-glucuronidase, CAT (chloramphenicol acetyl transferase; Alton and Vapnek (1979) Nature 282:864-869), luciferase, β-galactosidase and alkaline phosphatase (Toh, et al. (1980) Eur. J. Biochem. 182:231-238; and Hall et al. (1983) J. Mol. Appl. Gen. 2:101).

In these assays, cells harboring the reporter construct are contacted with a test compound. A test compound that either activates the promoter by binding to it or triggers a cascade that produces a molecule that activates the promoter causes expression of the detectable reporter. Certain other reporter assays are conducted with cells that harbor a heterologous construct that includes a transcriptional control element that activates expression of UCP-2 and a reporter operably linked thereto. Here, too, an agent that binds to the transcriptional control element to activate expression of the reporter or that triggers the

formation of an agent that binds to the transcriptional control element to activate reporter expression, can be identified by the generation of signal associated with reporter expression.

The level of expression or activity can be compared to a baseline value. As indicated above, the baseline value can be a value for a control sample or a statistical value that is representative of UCP-2 expression levels for a control population (e.g., healthy individuals not at risk for neurological injury such as stroke). Expression levels can also be determined for cells that do not express UCP-2 as a negative control. Such cells generally are otherwise substantially genetically the same as the test cells.

A variety of different types of cells can be utilized in the reporter assays. As stated above, certain cells are nerve cells that express an endogenous UCP-2. Cells not expressing UCP-2 can be prokaryotic, but preferably are eukaryotic. The eukaryotic cells can be any of the cells typically utilized in generating cells that harbor recombinant nucleic acid constructs. Exemplary eukaryotic cells include, but are not limited to, yeast, and various higher eukaryotic cells such as the COS, CHO and HeLa cell lines.

Various controls can be conducted to ensure that an observed activity is authentic including running parallel reactions with cells that lack the reporter construct or by not contacting a cell harboring the reporter construct with test compound. Compounds can also be further validated as described below.

C. Assays of UCP-2 Activity

Various screening methods can be conducted to identify compounds that increase the activity of UCP-2. Some of the UCP-2 activities that can be measured include determination of mitochondrial respiration and fatty acid transport rates as described supra in the section on diagnostic methods. The sequence homology between UCP-2 and UCP-1 indicates that under some conditions UCP-2 proton transport can be inhibited by certain purine nucleotides, such as diphosphate and triphosphate purine nucleotides. GDP, for instance, has be shown to be an inhibitor that binds to an inhibitory site on UCP-1 (see, e.g., Murdza-Inglis, D.L., et al. (1994) J. Biol. Chem. 269:7435-38; and Bouillaud, F., et al. (1994) EMBO J. 13:1990-97). Thus, screens to identify compounds that inhibit binding of such purine nucleotides either by binding to the same inhibitory site or at another site of UCP-2 can serve as potential activators of UCP-2. These type of compounds can be identified by using labeled-purine nucleotides, for example, and detecting the ability of test compounds to

inhibit binding of the labeled nucleotides to UCP-2 (e.g., UCP-2 containing mitochondrial membrane preparations). Assays based on measuring the mitochondrial membrane potential, and the associated protonmotive force (PMF), can be performed in both yeast and mammalian cells upon ectopic expression of UCP-2. Compounds that influence the PMF can be subsequently identified by fluorescent dyes or electrochemical methods.

Other assays can also be utilized in the screening process. Examples include assaying mitochondrial respiration rates, mitochondrial swelling and/or transport of fatty acids as described supra in the section diagnostic and prognostic methods. Regardless of the particular assay, various controls can be conducted to ensure that the observed activity is genuine. For example, assays can be conducted with cells that do not express UCP-2 or assays can be conducted in which cells that do express UCP-2 are not contacted with test compound.

D. Validation

Compounds that are initially identified by any of the foregoing screening methods can be further tested to validate the apparent activity. Preferably such studies are conducted with suitable animal models such as the rat model system described infra in Example 1. The basic format of such methods involves administering a lead compound identified during an initial screen to an animal that serves as a model for humans and then determining if UCP-2 is in fact upregulated. The animal models utilized in validation studies generally are mammals of any kind. Specific examples of suitable animals include, but are not limited to, primates, mice and rats.

Certain methods are designed to test not only the ability of a lead compound to increase UCP-2 activity in an animal model, but to provide protection after the animal has undergone transient ischemia for a longer period of time than shown to provide a protective effect. In such methods, a lead compound is administered to the model animal (i.e., an animal, typically a mammal, other than a human). The animal is subsequently subjected to transient ischemia for a period longer in duration than that shown to provide a protective effect. The conditions causing the ischemia are halted and UCP-2 activity monitored to identify those compounds still able to increase UCP-2 activity above a baseline level. Compounds able to enhance UCP-2 expression beyond the time period in which UCP-2 is upregulated in preconditioning models are good candidates for further study.

E. Compounds Affecting Mitochondria and Cell Apoptosis

Because of the evidence indicating that UCP-2 affects cellular apoptosis by altering mitochondrial permeability transition and membrane potential, as well as inhibiting activation of caspase-3 activation, screens can also be conducted to identify compounds that have similar effects on mitochondrial permeability transition, membrane potential and caspase-3 activation.

A variety of methods can be utilized to determine mitochondrial membrane potentials. One approach is to utilize fluorescent indicators(see, e.g., Haugland, 1996 Handbook of Fluorescent Probes and Research Chemicals, 6th ed., Molecular Probes, OR, pp. 266-274 and 589-594). Various non-fluorescent probes can also be utilized (see, e.g., Kamo et al. (1979) J. Membrane Biol. 49:105). Mitochondrial membrane potentials can also be determined indirectly from mitochondrial membrane permeability (see, e.g., Quinn (1976) The Molecular Biology of Cell Membranes, University Park Press, Baltimore, Maryland, pp. 200-217). Various ion sensitive electrode can also be utilized.

Caspase 3 activity can be monitored utilizing various known substrates known in the art. Suitable caspase-3 assays are described, for example, by (Ellerby et al., (1997) J. Neurosci. 17:6165; Rosen et al., (1997) J. Cell. Biochem. 64:50; and Kluck et al. (1997) Science 275:1132). Another caspase assay is described in Example 4 below.

Cytochrome c release from mitochondria can be detected using any of a number of immunological or spectroscopic methods.

F. Test Compounds

The screening methods can be conducted with essentially any type of compound potentially capable of activating UCP-2 expression. Consequently, test compounds can be of a variety of general types including, but not limited to, polypeptides; carbohydrates such as oligosaccharides and polysaccharides; polynucleotides; lipids or phospholipids; fatty acids; steroids; or amino acid analogs. The test compounds can be of a variety of chemical types including, but not limited to, heterocyclic compounds, carbocyclic compounds, β-lactams, polycarbamates, oligomeric-N-substituted glycines, benzodiazepines, thiazolidinones and imidizolidinones. Certain test agents are small molecules, including synthesized organic compounds.

Test agents can be obtained from libraries, such as natural product libraries or combinatorial libraries, for example. A number of different types of combinatorial libraries and methods for preparing such libraries have been described, including for example, PCT publications WO 93/06121, WO 95/12608, WO 95/35503, WO 94/08051 and WO 95/30642, each of which is incorporated herein by reference.

VI. Production of UCP-2

A. UCP-2 Nucleic Acids

UCP-2 nucleic acids can be obtained by any suitable method known in the art, including, for example: (1) hybridization of genomic or cDNA libraries with probes to detect homologous nucleotide sequences, (2) antibody screening of expression libraries to detect cloned DNA fragments with shared structural features, (3) various amplification procedures [e.g., polymerase chain reaction (PCR)] using primers that specifically hybridize to UCP-2 nucleic acids; and 4) direct chemical synthesis.

More specifically, UCP-2 nucleic acids can be obtained using established cloning methods. The nucleotide sequence of a gene or cDNA encoding UCP-2 (see, e.g., SEQ ID NO:1) is used to provide probes that specifically hybridize to a UCP-2 cDNA in a cDNA library, a UCP-2 gene in a genomic DNA sample, or to a UCP-2 mRNA in a total RNA sample (e.g., in a Southern or Northern blot). The libraries are preferably prepared from nerve cells. Once the target nucleic acid is identified, it can be isolated and cloned using well-known amplification techniques. Such techniques include, the polymerase chain reaction (PCR) the ligase chain reaction (LCR), Qβ-replicase amplification, the self-sustained sequence replication system (SSR) and the transcription based amplification system (TAS). Cloning methods that can be utilized to clone UCP-2 are described in Berger and Kimmel, Guide to Molecular Cloning Techniques, Methods in Enzymology, 152, Academic Press, Inc. San Diego, CA; Sambrook, et al. (1989) Molecular Cloning – A Laboratory Manual (2nd ed) Vols. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, NY; and Current Protocols (1994), a joint venture between Greene Publishing Associates, Inc. and John Wiley and Sons, Inc.

UCP-2 nucleic acids can also be obtained utilizing various amplification techniques. Such methods include, those described, for example, in U.S. Patent No. 4,683,202 to Mullis et al.; PCR Protocols A Guide to Methods and Applications (Innis et al.

eds) Academic Press Inc. San Diego, CA (1990); Kwoh et al. (1989) Proc. Natl. Acad. Sci. USA 86: 1173; Guatelli et al. (1990) Proc. Natl. Acad. Sci. USA 87: 1874; Lomell et al. (1989) J. Clin. Chem. 35: 1826; Landegren et al. (1988) Science 241: 1077-1080; Van Brunt (1990) Biotechnology 8: 291-294; Wu and Wallace (1989) Gene 4: 560; and Barringer et al. (1990) Gene 89: 117.

As an alternative to cloning a nucleic acid, a suitable nucleic acid can be chemically synthesized. Direct chemical synthesis methods include, for example, the phosphotriester method of Narang et al. (1979) Meth. Enzymol. 68: 90-99; the phosphodiester method of Brown et al. (1979) Meth. Enzymol. 68: 109-151; the diethylphosphoramidite method of Beaucage et al. (1981) Tetra. Lett., 22: 1859-1862; and the solid support method of U.S. Patent No. 4,458,066. Chemical synthesis produces a single stranded oligonucleotide. This can be converted into double stranded DNA by hybridization with a complementary sequence, or by polymerization with a DNA polymerase using the single strand as a template. While chemical synthesis of DNA is often limited to sequences of about 100 bases, longer sequences can be obtained by the ligation of shorter sequences. Alternatively, subsequences may be cloned and the appropriate subsequences cleaved using appropriate restriction enzymes. The fragments can then be ligated to produce a UCP-2 sequence.

Further specific guidance regarding the preparation of UCP-2 nucleic acids is provided by Fleury et al. (1997) Nature Genetics 15:269-272; Tartaglia et al., PCT Publication No. WO 96/05861; and Chen, et al., PCT Publication No. WO 00/06087, each of which is incorporated herein in its entirety.

B. UCP-2 Proteins

UCP-2 proteins can be produced through isolation from natural sources, recombinant methods and chemical synthesis. For example, UCP-2 proteins can be prepared by expressing cloned UCP-2 in a host cell. Cloned UCP-2 sequences are expressed in hosts after the sequences have been operably linked to an expression control sequence in an expression vector. Expression vectors are usually replicable in the host organisms either as episomes or as an integral part of the host chromosomal DNA.

Typically, the polynucleotide that encodes UCP-2 is placed under the control of a promoter that is functional in the desired host cell to produce relatively large quantities

of UCP-2. An extremely wide variety of promoters are well-known, and can be used in the expression vectors of the invention, depending on the particular application. Ordinarily, the promoter selected depends upon the cell in which the promoter is to be active. Other expression control sequences such as ribosome binding sites, transcription termination sites and the like are also optionally included. Constructs that include one or more of these control sequences are termed "expression cassettes." Expression can be achieved in prokaryotic and eukaryotic cells utilizing promoters and other regulatory egents appropriate for the particular host cell. Exemplary host cells include, but are not limited to, E. coli, other bacterial hosts, yeast, and various higher eukaryotic cells such as the COS, CHO and HeLa cells lines and myeloma cell lines.

Construction of suitable vectors containing one or more of the above listed components employs standard ligation techniques. A description of the preparation of the recombinant nucleic acids including sequences that encode UCP-2 can be found, for example, in Berger and Kimmel, Guide to Molecular Cloning Techniques, Methods in Enzymology, Volume 152, Academic Press, Inc., San Diego, CA (Berger); and Current Protocols in Molecular Biology, F.M. Ausubel et al., eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1998 Supplement) (Ausubel).

Once expressed, the recombinant polypeptides can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, ion exchange and/or size exclusivity chromatography, gel electrophoresis and the like (see, generally, R. Scopes, Protein Purification, Springer--Verlag, N.Y. (1982), Deutscher, Methods in Enzymology Vol. 182: Guide to Protein Purification., Academic Press, Inc. N.Y. (1990)).

As an option to recombinant methods, UCP-2 can be chemically synthesized. Such methods typically include solid-state approaches, but can also utilize solution based chemistries and combinations or combinations of solid-state and solution approaches. Examples of solid-state methodologies for synthesizing proteins are described by Merrifield (1964) J. Am. Chem. Soc. 85:2149; and Houghton (1985) Proc. Natl. Acad. Sci., 82:5132. Fragments of UCP-2 can be synthesized and then joined together. Methods for conducting such reactions are described by Grant (1992) Synthetic Peptides: A User Guide, W.H.

Freeman and Co., N.Y.; and in "Principles of Peptide Synthesis," (Bodansky and Trost, ed.), Springer-Verlag, Inc. N.Y., (1993).

Additional guidance specific for preparing UCP-2 proteins is provided by Fleury et al. (1997) Nature Genetics 15:269-272; Tartaglia et al., PCT Publication No. WO 96/05861; and Chen, et al., PCT Publication No. WO 00/06087.

VII. Variations

A. Synthesis of Analogs

Active test agents identified by the screening methods described herein that increase UCP-2 activity can serve as lead compounds for the synthesis of analog compounds. Typically, the analog compounds are synthesized to have an electronic configuration and a molecular conformation similar to that of the lead compound. Identification of analog compounds can be performed through use of techniques such as self-consistent field (SCF) analysis, configuration interaction (CI) analysis, and normal mode dynamics analysis. Computer programs for implementing these techniques are available. See, e.g., Rein et al., (1989) Computer-Assisted Modeling of Receptor-Ligand Interactions (Alan Liss, New York).

Once analogs have been prepared, they can be screened using the methods disclosed herein to identify those analogs that exhibit an increased ability to increase UCP-2 activity. Such compounds can then be subjected to further analysis to identify those compounds that appear to have the greatest potential as pharmaceutical agents. Alternatively, analogs shown to have activity through the screening methods can serve as lead compounds in the preparation of still further analogs, which can be screened by the methods described herein. The cycle of screening, synthesizing analogs and rescreening can be repeated multiple times.

B. Pharmaceutical Compositions

1. Composition

Compounds identified by the screening methods described above, analogs thereof and UCP-2 itself can serve as the active ingredient in pharmaceutical compositions formulated for the treatment of various neurological disorders including stroke. The compositions can also include various other agents to enhance delivery and efficacy. For instance, compositions can include agents capable of increasing the permeability of the

blood/brain barrier. Other agents that can be coadministered include anticoagulants and blood thinners. The compositions can also include various agents to enhance delivery and stability of the active ingredients.

Thus, for example, the compositions can also include, depending on the formulation desired, pharmaceutically-acceptable, non-toxic carriers of diluents, which are defined as vehicles commonly used to formulate pharmaceutical compositions for animal or human administration. The diluent is selected so as not to affect the biological activity of the combination. Examples of such diluents are distilled water, buffered water, physiological saline, PBS, Ringer's solution, dextrose solution, and Hank's solution. In addition, the pharmaceutical composition or formulation can include other carriers, adjuvants, or non-toxic, nontherapeutic, nonimmunogenic stabilizers, excipients and the like. The compositions can also include additional substances to approximate physiological conditions, such as pH adjusting and buffering agents, toxicity adjusting agents, wetting agents and detergents.

The composition can also include any of a variety of stabilizing agents, such as an antioxidant for example. When the pharmaceutical composition includes a polypeptide (e.g., UCP-2), the polypeptide can be complexed with various well-known compounds that enhance the *in vivo* stability of the polypeptide, or otherwise enhance its pharmacological properties (*e.g.*, increase the half-life of the polypeptide, reduce its toxicity, enhance solubility or uptake). Examples of such modifications or complexing agents include sulfate, gluconate, citrate and phosphate. The polypeptides of a composition can also be complexed with molecules that enhance their *in vivo* attributes. Such molecules include, for example, carbohydrates, polyamines, amino acids, other peptides, ions (*e.g.*, sodium, potassium, calcium, magnesium, manganese), and lipids.

Further guidance regarding formulations that are suitable for various types of administration can be found in Remington's Pharmaceutical Sciences, Mace Publishing Company, Philadelphia, PA, 17th ed. (1985). For a brief review of methods for drug delivery, see, Langer, Science 249:1527-1533 (1990).

2. Dosage

The pharmaceutical compositions can be administered for prophylactic and/or therapeutic treatments. Toxicity and therapeutic efficacy of the active ingredient can be determined according to standard pharmaceutical procedures in cell cultures and/or

experimental animals, including, for example, determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Compounds that exhibit large therapeutic indices are preferred.

The data obtained from cell culture and/or animal studies can be used in formulating a range of dosages for humans. The dosage of the active ingredient typically lines within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage can vary within this range depending upon the dosage form employed and the route of administration utilized.

3. <u>Administration</u>

The pharmaceutical compositions described herein can be administered in a variety of different ways. Examples include administering a composition containing a pharmaceutically acceptable carrier via oral, intranasal, rectal, topical, intraperitoneal, intravenous, intramuscular, subcutaneous, subdermal, transdermal, intrathecal, and intracranial methods.

For oral administration, the active ingredient can be administered in solid dosage forms, such as capsules, tablets, and powders, or in liquid dosage forms, such as elixirs, syrups, and suspensions. The active component(s) can be encapsulated in gelatin capsules together with inactive ingredients and powdered carriers, such as glucose, lactose, sucrose, mannitol, starch, cellulose or cellulose derivatives, magnesium stearate, stearic acid, sodium saccharin, talcum, magnesium carbonate. Examples of additional inactive ingredients that may be added to provide desirable color, taste, stability, buffering capacity, dispersion or other known desirable features are red iron oxide, silica gel, sodium lauryl sulfate, titanium dioxide, and edible white ink. Similar diluents can be used to make compressed tablets. Both tablets and capsules can be manufactured as sustained release products to provide for continuous release of medication over a period of hours. Compressed tablets can be sugar coated or film coated to mask any unpleasant taste and protect the tablet from the atmosphere, or enteric-coated for selective disintegration in the gastrointestinal tract. Liquid dosage forms for oral administration can contain coloring and flavoring to increase patient acceptance.

The active ingredient, alone or in combination with other suitable components, can be made into aerosol formulations (*i.e.*, they can be "nebulized") to be administered via inhalation. Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen.

Suitable formulations for rectal administration include, for example, suppositories, which consist of the packaged active ingredient with a suppository base. Suitable suppository bases include natural or synthetic triglycerides or paraffin hydrocarbons. In addition, it is also possible to use gelatin rectal capsules which consist of a combination of the packaged active ingredient with a base, including, for example, liquid triglycerides, polyethylene glycols, and paraffin hydrocarbons.

Formulations suitable for parenteral administration, such as, for example, by intraarticular (in the joints), intravenous, intramuscular, intradermal, intraperitoneal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives.

The components used to formulate the pharmaceutical compositions are preferably of high purity and are substantially free of potentially harmful contaminants (e.g., at least National Food (NF) grade, generally at least analytical grade, and more typically at least pharmaceutical grade). Moreover, compositions intended for in vivo use are usually sterile. To the extent that a given compound must be synthesized prior to use, the resulting product is typically substantially free of any potentially toxic agents, particularly any endotoxins, which may be present during the synthesis or purification process. Compositions for parental administration are also sterile, substantially isotonic and made under GMP conditions.

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The following examples are provided solely to illustrate in greater detail certain aspects of the invention and are not to be construed to limit the scope of the invention.

EXAMPLE 1

Differential Expression of UCP-2 in Preconditioning Rat Model

I. Background

Global cerebral ischemia of moderate duration causes selective neuronal degeneration in the hippocampus of rodent as well as human brains. Particularly sensitive are the pyramidal neurons in a sub-region of the hippocampus denoted CA1. This particular sensitivity to ischemia is an example of selective neuronal degeneration seen following global cerebral ischemia. The cell death process is a slowly evolving one that is not seen in the light microscope until 36-48 hrs of recovery following an ischemic insult of 10 minute duration. During this period, the neurons stay functional at least until 24 hours of recovery.

When a ten minute ischemic insult is preceded by a 3 minute ischemic insult in a defined time period, which by itself does not cause ischemic damage, no neuronal degeneration occurs in the CA1 region. This phenomenon is called ischemic preconditioning and is also seen in the heart and kidney, and possibly other organs.

II. Experimental/Results

A. Differential Expression of UCP-2

Rats of the same strain, age and sex were divided into two experimental groups: (a) animals in the first group, the "experimental group," underwent surgery including a single 3 minute bilateral occlusion of the carotid arteries to induce global ischemia under hypotension, and (b) animals in the second group were sham operated ("control group"). The sham operated animals were treated with cycloheximide and anesthetized, but not subjected to arterial occlusion. The animals were sacrificed 4 hours after the operation or being subjected to sham treatment and the CA1 region of their hippocampi were dissected. Poly-A+ RNA prepared from the collected tissues was converted into double-stranded cDNA (dscDNA). Subtractive hybridization was carried out using the dscDNA from preconditioned animals with an excess of dscDNA prepared from the sham operated animals. Differentially expressed gene fragments were cloned into a plasmid vector, and the resulting library was

transformed in E. Coli cells. Inserts of recombinant clones were amplified by the polymerase chain reaction (PCR). The PCR products (fragments of 200-2,000 bp in size) were sequenced using an oligonucleotide complementary to common vector sequences.

The differentially expressed fragments that were cloned included four independent clones: (a)SL3bE_F19 (SEQ ID NO:3); SL3bF_D20 (SEQ ID NO:4); SL3bC_M24 (SEQ ID NO:5); and SL3b_CP2_J11 (SEQ ID NO:6) whose expression level was found to be upregulated. The sequences determined for these clones were compared to public databases using the BLAST (blastn and tblastx) algorithm. The DNA sequence of these four clones was found to match the sequence for rat UCP-2 (Genbank ID:AB10743, Rattus norvegicus mRNA for UCP-2).

B. <u>In situ Hybridization</u>

A series of in situ hybridization experiments were conducted to confirm the CA1-specific upregulation of UCP-2 mRNA after ischemic preconditioning. In situ hybridization experiments were conducted on brain slices from the experimental and control rats using two different probes according to standard protocols [see, e.g., Harris, D. W. (1996) Anal. Biochem. 243:249-256; Singer, et al. (1986) Biotechniques 4:230-250; Haase et al. (1984) Methods in Virology, vol. VII, pp. 189-226; and Nucleic Acid Hybridization: A Practical Approach (Hames, et al., eds., 1987)]. The first probe, "oligo 28," had the sequence CTCTGGCAGGAACCCAGAGAACCGTGGAGTCAAACAGAGCCAGG (SEQ ID NO:7). The second probe, oligo 33, had the sequence AGAAGTGAAGTGGCAAGGGAGGTCGTCTGTCATGAGGTTGGCTT (SEQ ID NO:8).

The in-situ hybridization results are illustrated in Figs. 1A-1D (results with oligo 28) and Figs. 2A-2D (results with oligo 33). Using either oligo 28 or 33 as a probe, in rats under normal conditions UCP-2 mRNA expression is low in the CA1 region (sham; Figs. 1A and 2A). This expression increases at 2 days following 3 minute ischemia (3 min IPC; Figs. 1B and 2B). At one day after recovery following the 10 minute ischemia, i.e., approximately 12 hours prior to cell death, there is no increase in UCP-2 mRNA compared to controls (sh + 10 min ischemia; Figs. 1C and 2C). However, if the 10 minute ischemia period is preceded by a 3 minute episode of preconditioning, the expression of UCP-2 in the CA1 region increases (3 min IPC + 10 min ischemia; Figs. 1D and 2D). UCP-2 expression is highly expressed in the ischemia-resistant CA3 field of the rat hippocampus (Figs. 1A-1D),

as well as in the protected CA1 field after a combination of a 3 minute and 10 minute ischemic period (Fig. 1D). Thus, UCP-2 mRNA expression profile correlates well with the establishment of a neuroprotected state.

Utilizing in vivo and in vitro model systems of ischemic preconditioning, UCP-2 mRNA levels have been found to increase by approximately 1.5-fold in certain instances as measured using in-situ hybridization and cDNA. For quantification, in situ hybridization images were scanned and analyzed for pixel density in the CA1 field based on 5 independent in situ images. The pixel density in CA1 of sham (Fig 1 A, 2A) was 23 ±10, the pixel density for the 3 min ischemia + 48 h rec (Fig 1B, 2B) was 33±13. These results are statistically significant with a paired t-test value of $p<0.011$. As independent evidence, increase in UCP-2 mRNA expression levels have been also determined by cDNA arrays analysis: Recombinant inserts of the four UCP-2 containing clones (SL3bE_F19.Seq; SL3bF_D20.Seq; SL3bC_M24.Seq; and SL3b_CP2_J11.Seq) were arrayed on solid support and hybridized with labeled cDNA derived from CA1 regions of animals subjected to in vivo ischemic preconditioning and global ischemia, or with labeled cDNA derived from cortical neuronal cultures subjected to in vitro oxygen-glucose deprivation (see below). In both cases, compared to control CA1 regions or control cultures, a 1.5-fold increase in UCP-2 mRNA abundance was observed.

C. Western Blot Analysis of UCP-2 Expression

UCP-2 protein levels in rat preconditioned primary cortical neuronal cultures were analyzed with western blot. Primary cortical cell cultures were prepared from gestational day 17 fetal rats. Briefly, the cortex was dissected under a microscope, and the cells dissociated by trituration in modified Eagle's medium (MEM), 10% horse serum, 10% fetal bovine serum, 2 mM glutamine following a 30 min digestion in 0.027% trypsin/saline solution (Gibco BRL, Gaithersburg, Maryland). Cells were plated in 15 mm multiwell (Nunc) plates coated with polyornithine at a density of 3-4 x 10^5 cells per well. Four days after plating, the cells were treated with 10 µg/ml of 5-fluoro-2'-deoxyuridine for 3 days to inhibit proliferation of non-neuronal cells. Cultures were maintained in MEM, 5% horse serum, 2 mM glutamine in 8% CO_2, humidified, 37°C atmosphere. The medium was changed twice a week. Mature neurons (14 days in culture) were used for all experiments. Combined oxygen-glucose deprivation was performed by complete exchange of media with

deoxygenated, glucose-free Earle's balanced salt solution (EBSS) containing 116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, and 0.9 mM $CaCl_2$, bubbled with 5% H_2/85% N_2/5% CO_2. The cultures were kept in an anaerobic chamber for 5 or 60 min containing the gas mixture. OGD was terminated by removal of the cultures from the chamber and replacement of the EBSS solution with oxygenated growth media.

Increased levels of UCP-2 protein were observed in the cortical neuronal cultures preconditioned by exposure either to OGD for 10 min or to 40μM NMDA for 5 min. The results are shown in Fig. 3.

The foregoing results demonstrate that there is an inverse correlation between an increased expression of UCP-2 and neuronal damage. Said differently, these results show that the expression pattern of UCP-2 correlates with a neuroprotected state of the brain during and following ischemia. These results indicate that agents that can increase the activity of UCP-2 are neuroprotective and can serve as therapeutic agents against stroke and other neurodegenerative diseases.

EXAMPLE 2

Neuroprotection by Overexpression of UCP-2 in Neurons via a Recombinant UCP-2 Adenovirus

I. Methods

A. Generation of Recombinant UCP-2 Adenovirus

Whole rat brain cDNA was used to obtain a PCR fragment containing a 930 nucleotide ORF (Open Reading Frame) corresponding to rat UCP-2. The PCR product was ligated to the T/A cloning vector pCR2.1 (Invitrogen, San Diego) and sequenced. Rat UCP-2 cDNA was then subcloned into the pShuttle-CMV transfer vector (Quantum Biotechnologies, Montreal). The resulting plasmid was linearized with Pme I and co-transformed into *E. coli* strain BJ183 together with pAdEasy-1, the viral DNA plasmid (Quantum Biotechnologies, Montreal). The pAdEasy-1 is E1 and E3 deleted, and its E1 functions can be complemented in 293 cells. Recombinants were selected with kanamycin and screened by restriction enzyme analysis. The recombinant adenoviral construct was then cleaved with PacI to expose its ITR (Inverted Terminal Repeats) and transfected into 293 cells to produce viral particles. A stock of 1012 VP/ml (Viral Particles per ml) of recombinant UCP-2 adenovirus

was produced. Expression of recombinant rat UCP-2 driven by the strong CMV (CytoMegalo Virus) promoter was confirmed in infected 293 cells by Western blot analysis.

B. In vitro Functional Validation of UCP-2

Cell Culture. Primary cortical neuronal cultures were prepared from gestational day 17 fetal rats. The cortex was dissected under a microscope, and the cells dissociated by trituration in modified Eagle's medium (MEM), 10% horse serum, 10% fetal bovine serum, 2 mM glutamine following a 30 min digestion in 0.027% trypsin/saline solution. Cells were plated in 6 mm multiwell (Nunc) plates coated with polyornithine at a density of 1-2 x 105 cells per well. Four days after plating, the cultures were treated with 10 µg/ml of 5-fluoro-2'-deoxyuridine for 3 days to inhibit proliferation of non-neuronal cells. Cultures were maintained in MEM, 5% horse serum, 2 mM glutamine in 8% CO_2, humidified, 37 °C atmosphere. The medium was changed twice a week. Mature neurons (12 days in vitro, DIV12) were used for all experiments. In mature cultures, neurons represent 70-90% of the total number of cells.

Infection of Neurons. Neurons were exposed to 2 x 1010 VP/ml (an approximate multiplicity of infection of 20 virus particles per cell) of recombinant adenovirus containing rat UCP-2. An adenovirus expressing the *lacZ* gene driven by the same promoter (CMV) was used as control. DIV12 neuronal cultures were exposed to recombinant adenovirus in serum-free medium for 2 h at 37 °C. During this incubation, cells were rocked gently every 15 minutes. After 2h in serum-free medium, regular growth medium containing 5% horse serum was added to the cultures. Experiments were performed on infected cells 24 h after exposure to viral particles.

Ischemia. Combined oxygen-glucose deprivation (OGD) was performed 24 h after adenoviral infection of neurons by complete exchange of media with deoxygenated, glucose-free Earleís balanced salt solution (EBSS) containing 116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, and 0.9 mM $CaCl_2$, bubbled with 5% H_2/85% N_2/5% CO_2. The cultures were kept in an anaerobic chamber for 90 min containing the gas mixture, 5% H_2/85% N_2/5% CO_2, maintained at 37 C. OGD was terminated by removal of the cultures from the chamber and replacement of the EBSS solution with oxygenated growth media.

Survival/Death Assay. Neuronal survival was assessed 24 h after OGD and presented as % cell death determined as the ratio of dead to live cells. Cell viability was

determined by determination of fluorescence readings by a fluorescence plate reader after staining the nuclei of dead neurons with 8 μM of Ethidium Homodimer, and the cytoplasm of alive cells by Calcein. For some treatment conditions, cell survival results were confirmed by an independent method using computer assisted cell counting after staining of all nuclei with 1 μg/ml Hoescht 33342 and dead cell nuclei with 7 μM propidium iodide. At least three independent experiments utilizing three separate wells per experiment were performed.

Western Blot Analysis. UCP-2 expression in rat primary neuronal cultures was analyzed by western blot. A total of 60 μg protein from each fraction was resolved on a 10-20% acrylamide gradient gel, blotted on nitrocellulose membrane and developed with an anti-UCP2 antibody, or anti MAP-2 antibody.

Immunofluorescence Analysis. Primary neurons were cultured on PDL coated coverslips and infected with Ad.UCP-2. A mitochondria-selective probe, MitoTracker green (250 nM, Molecular Probe, Eugene, OR), was used for staining cells for 30 min at 37° C in culture medium. After incubation with MitoTracker green, cells was washed in PBS, fixed with fresh made 4% paraformaldehyde in phosphate buffer solution (PBS) for 30 min at 37°C and followed by permeabilization with 0.1% Triton X-100 for 3 min at 4°C. Fixed cells were blocked with 5% BSA, 0.1% Tween 20 in PBS for 1 hr at room temperature and incubated overnight at 4° C with a monoclonal antibody (1:100, CALBIOCHEM, CA) that recognizes UCP-2. Following two washes with PBS, primary antibody was detected using Cy™3-conjugated anti-rabbit Ig G (1:100 Jackson ImmunoResearch Laboratories, Inc. PA). Neurons were mounted and examined with fluorescent microscope at magnification of 100 X under oil immersion. Images were obtained with digital video camera system (Optronics, Goleta, CA).

II. <u>Results</u>

Fig. 4 shows results of western blot analysis of UCP-2 protein levels in rat primary cortical neuronal cultures. Twenty-four hours after infection with the recombinant adenovirus, UCP-2 protein levels were increased approximately 20-fold in Ad.UCP-2 exposed cultures compared to Ad.lacZ infected cultures. Probing with an anti-MAP2 antibody demonstrates equal protein loading.

Results of immunofluorescence analysis of UCP-2 recombinant expression in rat primary neuronal cultures are shown in Fig. 5. Cultures were infected with recombinant

UCP-2 adenovirus and stained with an UCP-2 specific antibody (UCP-2), or a mitochondria-selective dye (MitoTracker). Images were taken at 10X and 100X magnification. The staining pattern indicates localization of UCP-2 protein in mitochondria.

The effect of UCP-2 overexpression on neuronal death induced by OGD is shown in Figs. 6A and 6B. To control against effects induced by the recombinant adenovirus system, the reporter *lacZ* gene was used. OGD induced over 90 % neuronal death in cultures infected with *lacZ* adenovirus and in non-infected control cells (Fig. 6A). In contrast, neurons expressing recombinant UCP-2 were remarkably protected from OGD-induced damage since OGD caused death in only 48% of these cells. These results show that overexpression of UCP-2 confers 55-60% protection to neurons against OGD-induced death, suggesting a major role of UCP-2 as a neuroprotective protein against ischemia.

UCP-2 overexpression in cortical neuronal cultures confers a level of resistance (52% protection) to OGD-induced cell death that is very similar to the protection induced by ischemic preconditioning in vitro (59% protection) (Fig. 6B).

The above results indicate that UCP-2 and ischemic preconditioning may trigger common neuroprotective pathways, and further support the remarkable neuroprotective action of UCP-2 against neuronal loss induced by stroke, global ischemia and possibly other neuronal insults.

EXAMPLE 3

Reduction of Ischemic Brain Damage In Vivo by UCP-2 Overexpression

To examiner effect of UCP-2 overexpression on ischemic brain damage in vivo, focal ischemia in mouse was induced by insertion of a 6-nylon suture coated with silicone into the middle cerebral artery under 2% halothane in a mixture of N_20 /O_2 (70/30) as described in Hara et al., *J. Cereb. Blood Flow Metab.* **16**, 605 (1996), while the animal was spontaneously breathing through an open mask. The body temperature was kept at 36-38° C, and cortical blood flow was measured by laser Doppler flowmetry. Following 50 min occlusion, the nylon suture was removed. The wounds were sutured, and the animal was allowed to recover for 24 hr before being sacrificed. The brain was sectioned and stained red when tetrazolium trichloride is oxidized by mitochondria in viable tissue. The infarct was calculated as described in Kuroda et al., *J Cereb Blood Flow Metab* 19, 778 (1999).

Fig. 7 shows ischemic brain damage induced by occlusion of the middle cerebral artery in wild-type mice (wt) and UCP-2/3 transgenic mice (UCP-2/3 Tg). White areas in the top panel indicate tissue damage. The data in the bottom panel represent the mean infarct size in mm3 ± SEM (Mann Whitney U test. n=9). The results demonstrate that UCP-2 overexpression results in smaller infract size, indicating reduced ischemic brain damage *in vivo*.

EXAMPLE 4

UCP-2 Regulation of Mitochondrial Permeability Transition, Release of Cytochrome C, and Caspase 3 Activation

I. Methods

Isolation of brain mitochondria and analysis. Rat brain mitochondria were isolated as described in Xiong et al., *J. Neurotrauma*. 14, 23 (1997) with some modifications. Mouse cortex was homogenized in 320 mM sucrose, 1 mM EGTA, 10 mM Tris (pH 7,4) using a 2 ml Kontes Teflon homogenizer. The debris was then pelleted (2.000 g, 3 min, 4°C). The supernatant was centrifuged (10.000 g, 10 min, 4°C). The crude mitochondrial pellet was washed once in homogenization buffer. Respiratory activity of mitochondrial preparations was determined as described in Sims, *J. Neurochem*. 55, 698 (1990). Generation of free radical by mitochondria was measured using flow cytometric analysis in a FACSCalibur equipped with a 488 nm argon laser. The concentrations of fluorescent probes used did not affect RCR compared to control.

For analysis, mitochondria were suspended in 500 µl buffer (250 mM sucrose, 20 mM MOPS, 10 mM Tris-Base, 100 µM P_i(K), 0,5 mM Mg^{2+}, pH 7,0) which also contains 5 mM succinate. To assess purity of the analysed gate, mitochondria were stained with 100 nM 10-nonyl-acridine orange (Mol. Probes, Oregon, USA). This gate was used for analysis of free radical generation by H2DCF-DA analysis (Teranishi *et al.*, *Exp. Mol. Pathol.* 68, 104 (2000). A total of 40 µg mitochondrial protein was resuspended in 500 µl of analysis buffer containing 5 mM succinate, 1 µM CsA either in the presence or in the absence of 10 µM H2DCFDA. H2DCFDA is converted to the fluorescent compound DCF upon reaction with ROS. The geometric mean fluorescence for each sample was calculated, and the background fluorescence for each sample was subtracted. The resulting value was compared between the

two genotypes at different time points. Samples were analyzed in triplicate. At each time point, the mean fluorescence for each sample was calculated, and the control value (background) was subtracted.

Analysis of mitochondrial membrane potential. Rat primary neuronal cultures were incubated with 2μg/ml of JC-1 (Molecular Probes) for 20 min at 37C. Following incubation, cells were washed twice with 1X PBS and observed under a fluorescence microscope using a 475 nm excitation optical filter.

Western blot analysis of mitochondria proteins. Cytosolic and mitochondrial extracts from cultured rat primary cortical neurons were prepared using the ApoAlert Cell Fractionation Kit (Clontech) according to the manufacturer's instructions. A total of 60 μg protein from each fraction was resolved on a 10-20% acrylamide gradient gel, blotted on nitrocellulose membrane and developed with an anti-cytochrome c antibody (1:500, BD Pharmingen).

Caspase-3 activity assay. Caspase-3 activity assay was employed to study effect of overexpression of UCP-2 on apoptotic cascade. Caspase-3 activity was measured in whole cell lysates of cultured rat primary cortical neurons using the Caspase Assay Kit (Clontech) according to the manufacturer's instructions. The caspase-3 activation assay provides a simple means for assaying caspase-3 activity in mammalian cells. In this assay a fluorescent emission shift of 7-amino-4-trifluoromethyl coumarin (AFC) is detected after cleavage of the AFC-substrate conjugate by caspase 3. DEVD-AFC, usually emits blue light (λmax =400 nm). However, upon proteolytic cleavage of the substrate by caspase 3, free AFC fluoresces yellow-green at 505 nm. Generation of a recombinant UCP-2 adenovirus, cell culture conditions, infection of neuron cells, inducement of OGD and survival/death assays were performed as described in Example 2.

II. <u>Results</u>

Isolated brain mitochondria were analyzed by flow cytometry as shown in Fig. 8. The upper left panel shows results plotted for side scattering (SSC) and forward scattering (FSC). Each dot represents one particle, and particles within area R1 were selected for analysis. Samples were stained with 100 nM 10-nonyl-acridine orange (NAO), a mitochondrial (cardiolipin) dye. As shown in the upper right panel, more than 98% of the events within gate R1 stain for NAO (right peak), suggesting that mainly mitochondria were

analyzed (upper right:). The left peak in the upper right panel of Fig. 8 represents background sample fluorescence. The lower left panel of Fig. 8 shows generation of reactive oxygen species (ROS) in mitochondria isolated from cortex of UCP-2/3 Tg animals (middle peak) and wt animals (right peak) measured as formation of dichlorofluorescein (DCF) from H2DCF-diacetate. The left peak in this panel shows background fluorescence. The lower right panel shows the generation of ROS in brain cortex mitochondria from UCP-2/3 Tg animals (squares, dashed line) and wt animals (triangles, solid line) at 5 time points of incubation with H2DCF (lower right). The values were compared using an unpaired student's t-test (* = $p<0.05$).

The results indicate that overexpression of UCP-2 blocks OGD-induced mitochondrial membrane depolarization in rat primary cortical neuronal cultures. The cultures were infected with Ad.UCP-2 or Ad.lacZ, and 24 h later subjected to 90 min of OGD. Changes in the mitochondrial membrane potential were qualitatively assessed using the cationic fluorescent dye JC-1 at 8 h after OGD. Compared to Ad.lacZ infected cultures, cultures expressing Ad.UCP-2 displayed a higher red/green fluorescence ratio (data not shown), indicating preservation of the mitochondrial membrane potential.

Results of western blot analysis of cytochrome c protein levels in cytosolic and mitochondrial cell fractions are shown in Fig. 9. Rat primary cortical cultures were infected with Ad.UCP-2 or Ad.GFP, and 36 h later subjected to 90 min of OGD. Cytosolic (C) and mitochondrial (M) fractions were prepared 24 h after exposure to OGD, adjusted for protein concentration and subjected to immunoblot analysis using a cytochrome c specific antibody. The presence of cytochrome c in the cytosolic fraction after 90 min of OGD of Ad.GFP infected cells indicates that cell death involves mitochondrial release of cytochrome c into the cytosol. The bottom panel shows quantification of the western blot results and provides ratio of cytochrome c band intensity in the mitochondrial versus the cytosolic fraction for each condition. A lower ratio indicates release of cytochrome c from mitochondria into cytosol. The results indicate that UCP-2 overexpression reduces OGD-induced cytochrome c release from mitochondria. A total of 3 independent experiments showed similar results. Western blot results were confirmed by quantitative ELISA analysis using a Quantikine M Immunoassay kit from R&D Systems.

Activation of caspase 3 is a marker for apoptosis. To elucidate the mechanism by which UCP-2 generates its neuroprotective effect, tests were conducted to determine what

effect overexpression of UCP-2 had on components of an apoptotic cascade. In particular, the effect of UCP-2 overexpression on the activity of caspase 3 was determined. This was done by testing the effect of UCP-2 overexpression on neuronal death induced by OGD using a recombinant adenovirus to achieve overexpression of UCP-2. An recombinant adenovirus having the *lacZ* gene instead of UCP-2 was used as a control.

Fig. 10 shows that overexpressed UCP-2 in cortical cultures blocks OGD-induced caspase 3 activation. The results strongly indicate that UCP-2 is neuroprotective by inhibition of apoptosis, including the blockade of caspase 3 activation by UCP-2. Programmed cell death, or apoptosis, requires activation of a series of cysteine proteases that specifically cleave target proteins after an aspartate residue.

***

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes to the same extent as if each individual publication, patent or patent application were specifically and individually indicated to be so incorporated by reference.

WHAT IS CLAIMED IS:

1. A method for diagnosing occurrence of a stroke or assessing a patient's susceptibility to a stroke, the method comprising detecting in a patient sample an elevated level of UCP-2 expression.

2. The method of claim 1, wherein detection comprises detecting an elevated level of UCP-2 transcript.

3. The method of claim 2, wherein detection comprises probing the sample with a nucleic acid probe that is homologous to at least 15 consecutive nucleotides of a UCP-2 sequence and determining the amount of nucleic acid bound by the probe.

4. The method of claim 1, wherein detection comprises detecting an elevated level of a UCP-2 polypeptide.

5. The method of claim 4, wherein detection comprises assaying for the presence of the UCP-2 polypeptide by contacting the sample with an antibody that specifically binds to the UCP-2 polypeptide to form a complex and detecting the complex.

6. The method of claim 5, wherein detection comprises performing an ELISA.

7. The method of claim 1, wherein the stroke is an ischemic stroke.

8. A method for assessing a patient's risk of having a stroke comprising comparing the level of UCP-2 expression in a test sample from the patient with a baseline value, wherein an elevated level of UCP-2 expression in the patient sample relative to the baseline indicates that the patient is at risk for stroke.

9. The method of claim 8, wherein the baseline value is the level of UCP-2 expression in a patient sample obtained prior to the test sample.

10. The method of claim, 8, wherein the baseline value is an average or mean value for UCP-2 expression in a population of control individuals.

11. A method for treating a subject having or being susceptible to a neurological disorder or a neuronal injury, the method comprising administering to the subject an effective amount of an agent that increases the activity of UCP-2.

12. The method of claim 11, wherein the neuronal injury is a stroke.

13. The method of claim 12, wherein the neuronal injury is an ischemic stroke.

14. The method of claim 11, wherein the neurological disorder is selected from the group consisting of Parkinson's disease, Huntington's disease, inherited ataxias, motor neuron diseases, Alzheimer's disease, epilepsy, and traumatic brain injury.

15. The method of claim 11, wherein the subject is susceptible to the neurological disorder or the neuronal injury, and the subject is administered a prophylactic amount of the agent prior to occurring of the neurological disorder or the neuronal injury.

16. The method of claim 11, wherein the subject has the neurological disorder or the neuronal injury, and the subject is administered a therapeutic amount of the agent.

17. The method of claim 11, wherein the agent is administered in combination with a secondary agent that increases the permeability of the blood/brain barrier.

18. The method of claim 17, wherein the secondary agent is selected from the group consisting of bradykinin, serotonin, histamine and arachidonic acid.

19. The method of claim 11, wherein the agent is administered in combination with an anticoagulant.

20. The method of claim 11, wherein the agent is a purified UCP-2 polypeptide in combination with a pharmaceutically acceptable carrier.

21. The method of claim 11, wherein the agent is an agent other than UCP-2.

22. The method of claim 21, wherein the agent stimulates the synthesis or expression of UCP-2 or any other UCP-2 inducer.

23. The method of claim 21, wherein the agent comprises a nucleic acid that encodes UCP-2 or any other UCP-2 inducer.

24. The method of claim 23, wherein the agent comprises a vector that contains the nucleic acid that encodes UCP-2.

25. The method of claim 24, wherein the vector is a viral vector.

26. The method of claim 25, wherein the viral vector is an adenoviral vector.

27. The method of claim 26, wherein the vector further comprises a promoter operably linked with the nucleic acid that encodes UCP-2, the promoter selectively driving expression of UCP-2 or a UCP-2 inducer in nerve cells.

28. The method of claim 27, wherein the nerve cells are cortical neuron cells, hippocampal neuron cells or neuronal cells in any other brain region affected by a stroke.

29. The method of claim 25, wherein the viral vector is introduced into the cerebrospinal fluid.

39. The method of claim 37, wherein the neuronal injury is ischemic stroke.

40. The method of claim 37, wherein the neurological disorder is selected from the group consisting of Parkinson's disease, Huntington's disease, inherited ataxias, motor neuron diseases, Alzheimer's disease, epilepsy, and traumatic brain injury.

41. The method of claim 37, wherein the agent inhibits the loss of mitochondrial membrane potential.

42. The method of claim 37, wherein the agent inhibits cytochrome c release from mitochondria.

43. The method of claim 37, wherein the agent inhibits caspase 3 activation.

30. The method of claim 25, wherein the viral vector is introduced into the intraventricular space.

31. The method of claim 24, further comprising producing ex vivo genetically-modified neuronal or non-neuronal stem cells that harbor a vector that includes a nucleic acid encoding for UCP-2, and wherein administering comprises introducing the modified stem cells into the intracerebroventricular space or into the cerebrospinal fluid.

32. A method for screening for an agent useful for treating a neurological disorder or a neuronal injury, the method comprising identifying an agent that upregulates UCP-2 expression and/or activity.

33. The method of claim 32, wherein the neuronal injury is stroke.

34. The method of claim 32, wherein the neurological disorder is selected from the group consisting of Parkinson's disease, Huntington's disease, inherited ataxias, motor neuron diseases, Alzheimer's disease, epilepsy, and traumatic brain injury.

35. The method of claim 32, wherein the method further comprises:

(a) administering to a test subject a test compound, wherein the test subject is a mammal other than a human;

(b) preconditioning the test subject; and

(c) determining in a sample from the test subject the expression level of UCP-2 to identify a test agent that upregulates UCP-2 expression in the test subject.

36. The method of claim 35, wherein the test and control subject is a rat.

37. A method of screening for an agent useful for treating a neurological disorder or a neuronal injury, the method comprising identifying an agent that inhibits cellular apoptosis.

38. The method of claim 37, wherein the neuronal injury is stroke.

First Named Inventor	:	Michael E. Spurlock	
Appln. No.	:	09/928,522	
Filed	:	August 13, 2001	Group Art Unit: 1647
Title	:	Bovine Leptin Protein, Antisense and Antibody	Examiner: C. J. Saoud
Docket No.	:	LL31.12-0015	

EXHIBIT M

of

DECLARATION

submitted under 37 C.F.R. 1.132

U.S. 2004 0137492A1

First Named Inventor	:	Michael E. Spurlock	
Appln. No.	:	09/928,522	
Filed	:	August 13, 2001	Group Art Unit: 1647
Title	:	Bovine Leptin Protein, Antisense and Antibody	Examiner: C. J. Saoud
Docket No.	:	LL31.12-0015	

EXHIBIT K

of

DECLARATION

submitted under 37 C.F.R. 1.132

WO 02/036829A2

oligo 28

A B C D

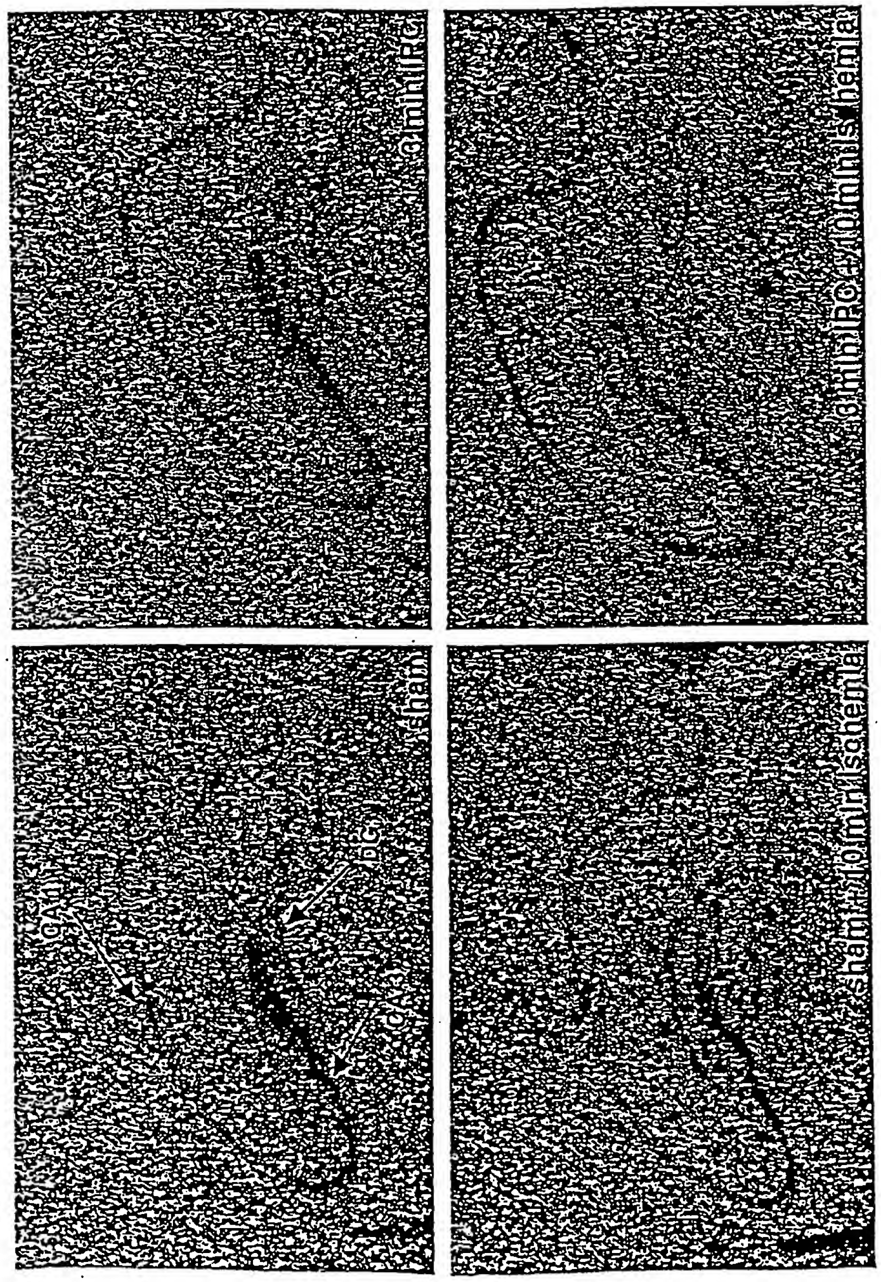


FIG. 1

oligo 33

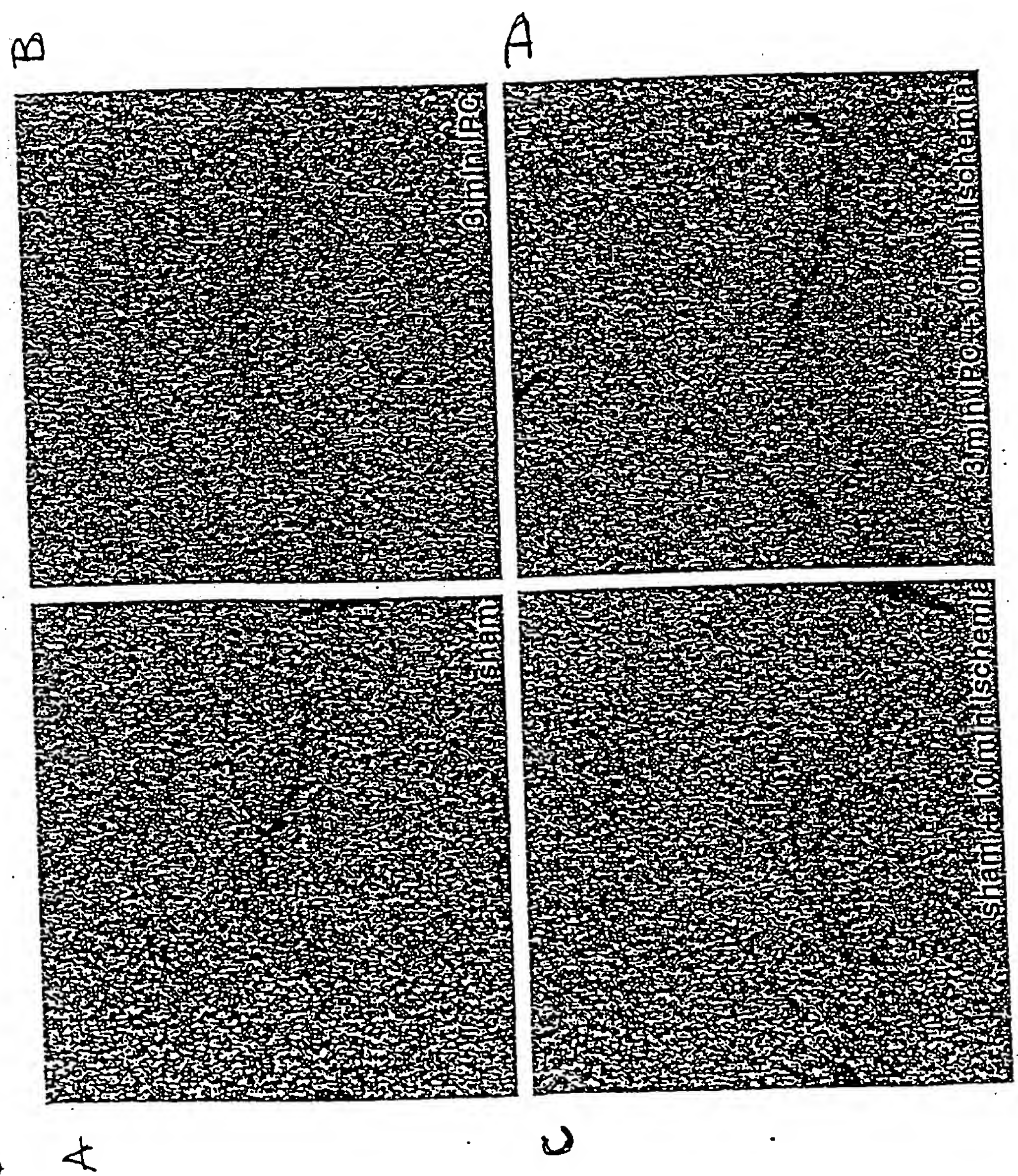


FIG. 2

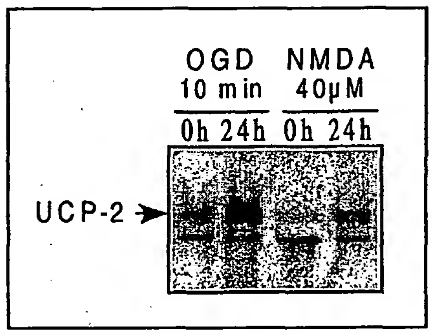


FIG. 3

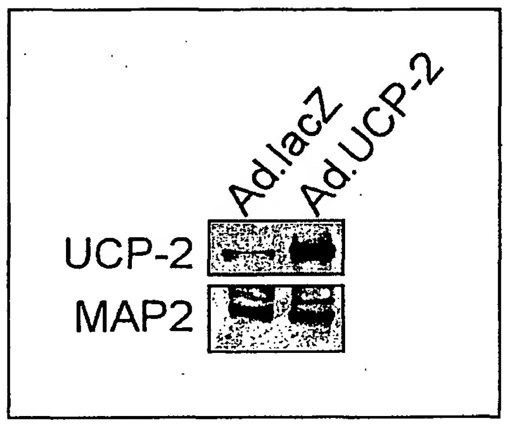


FIG. 4

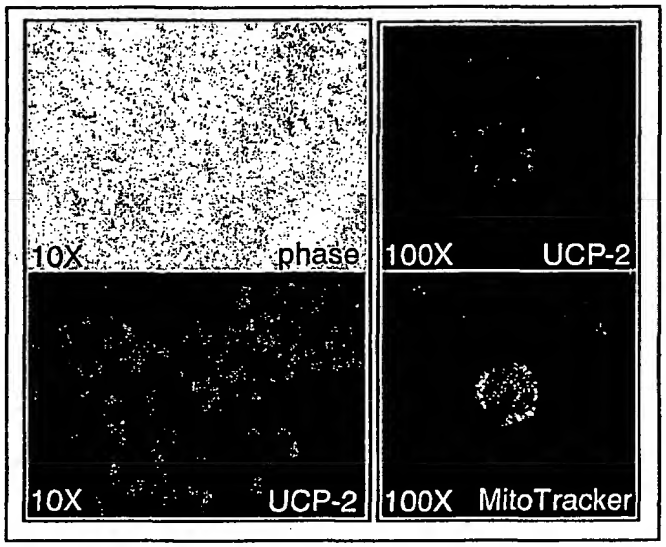
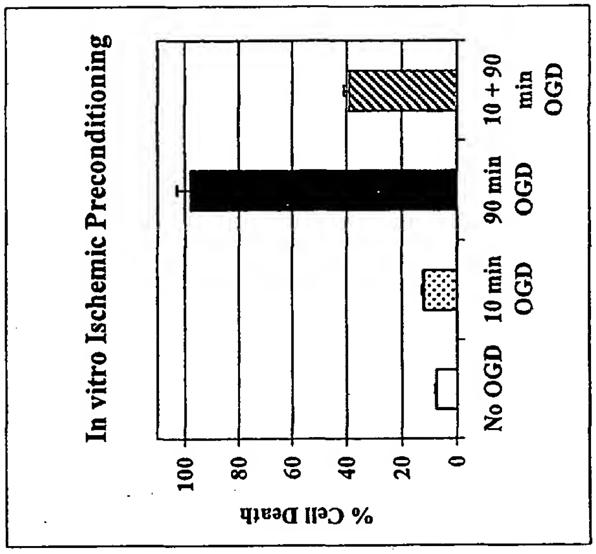


FIG. 5


In vitro Ischemic Preconditioning
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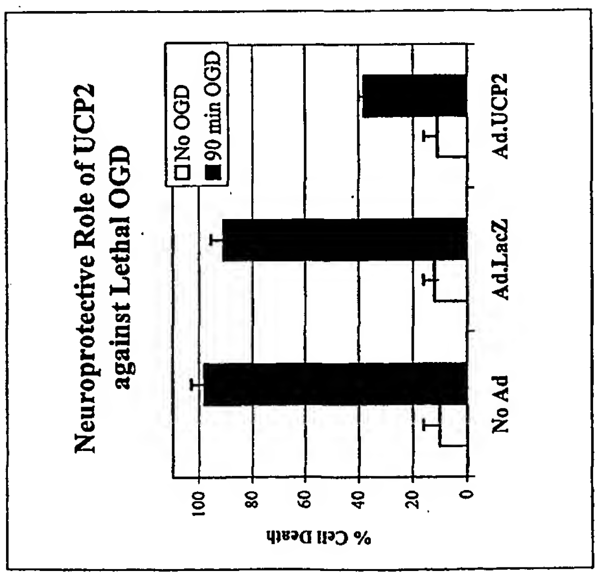
80
60
40
20
0
No OGD
10 min OGD
90 min OGD
10 + 90 min OGD

Fig. 6B


Neuroprotective Role of UCP2 against Lethal OGD
% Cell Death
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80
60
40
20
0
No OGD
90 min OGD
No Ad
Ad.LacZ
Ad.UCP2

Fig. 6A

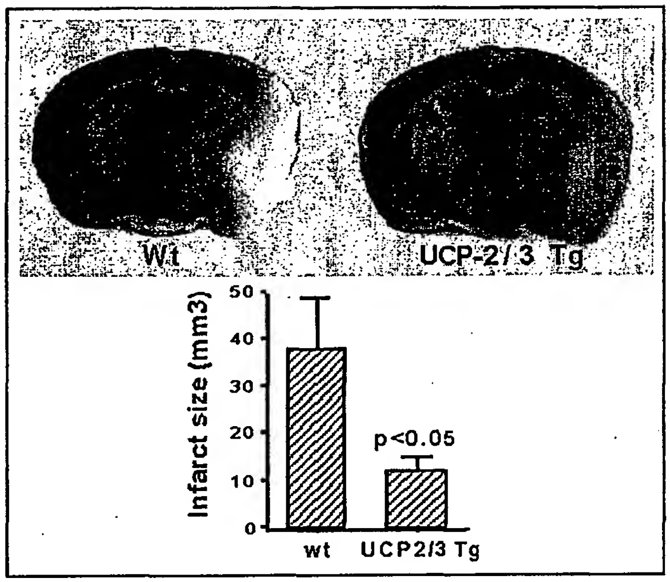


FIG. 7

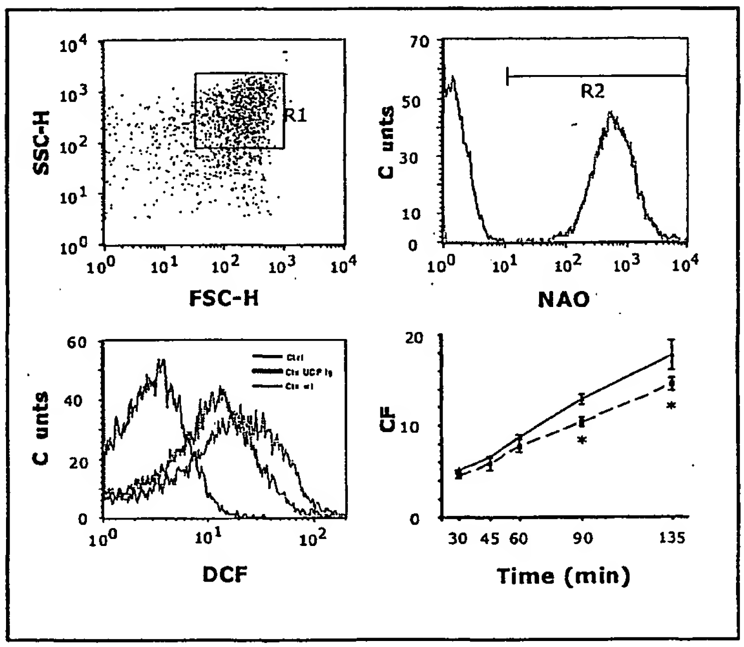
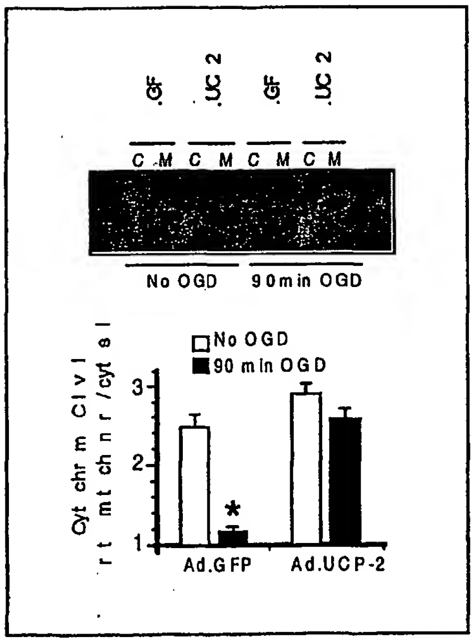


FIG. 8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.GF
.UC 2
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2
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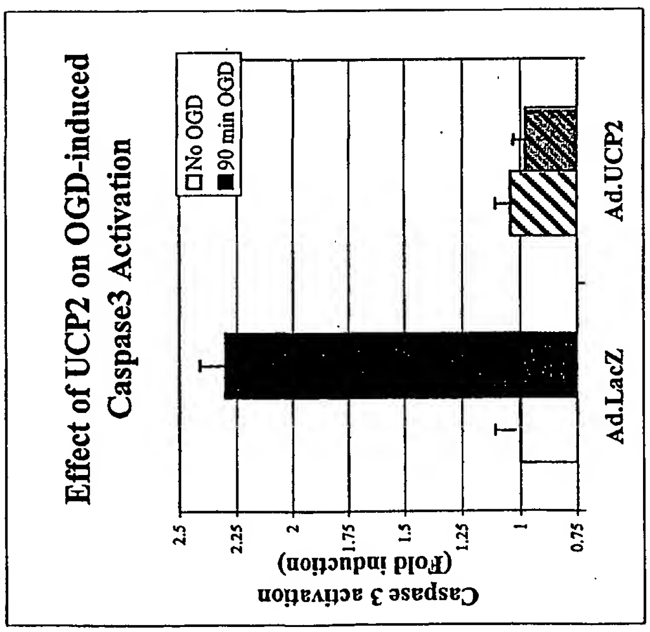
*
Ad.GFP
Ad.UCP-2

FIG. 9

Fig. 10


Effect of UCP2 on OGD-induced
Caspase3 Activation
Caspase 3 activation
(Fold induction)
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2.25
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1.5
1.25
1
0.75
No OGD
90 min OGD
Ad.LacZ
Ad.UCP2

SEQUENCE LISTING

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TGGAGGCCTCAGCGTGAGACCTCAAAGCACCCTCCCGACTCC
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SEQ ID NO:5 (Clone SL3bC_M24)

CGGCCGAGGTGCCGTCTGCACTCCTGTGTTCTCCTGTGTATTCTCCTGCGGTCCGGACACAA
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TGGGACAGC

SEQ ID NO:6 (Clone SL3b_CP2_J11)

CGGGCAGGTACTGGGCGCTGGCGGCGGTGCGCGCTAGCCCTTGACTCTCTCCTTGGATCTGC
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AGCCCCCAGGAACTTCACGGTGGCTGTGGGGGGCACATCGGTGGCCTTGAAACCAACCATGA
TTCTGACTTCCTGCTACCTCCCAGA

SEQ ID NO:7 (Oligo 28)

CTCTGGCAGGAACCCAGAGAACCGTGGAGTCAAACAGAGCCAGG

SEQ ID NO:8 (Oligo 33)

AGAAGTGAAGTGGCAAGGGAGGTCGTCTGTCATGAGGTTGGCTT

First Named Inventor	:	Michael E. Spurlock	
Appln. No.	:	09/928,522	
Filed	:	August 13, 2001	Group Art Unit: 1647
Title	:	Bovine Leptin Protein, Antisense and Antibody	Examiner: C. J. Saoud
Docket No.	:	LL31.12-0015	

EXHIBIT J

of

DECLARATION

submitted under 37 C.F.R. 1.132

U.S. PATENT NO. 6,156,546

First Named Inventor	:	Michael E. Spurlock	
Appln. No.	:	09/928,522	
Filed	:	August 13, 2001	Group Art Unit: 1647
Title	:	Bovine Leptin Protein, Antisense and Antibody	Examiner: C. J. Saoud
Docket No.	:	LL31.12-0015	

EXHIBIT F

of

DECLARATION

submitted under 37 C.F.R. 1.132

Hyperdictionary @ www.hyperdictionary.com/dictionary/Denhardt's + solution; obtained from the Internet on November 12, 2004

hyperdictionary

English Dictionary Computer Dictionary Thesaurus Dream Dictionary Medical Dictiona

Search Dictionary: Denhardt's solution Search

DENHARDT'S SOLUTION: Dictionary Entry and Me

Biology Dictionary

Definition: A solution commonly used during probe hybridizations that involve filters (such as Southern, No Western blots). The solution contains ficoll, bovine serum albumin, polyvinylpyrrolidone (PVP), a concentration of nonspecific DNA so the probe won't hybridize nonspecifically.

HOME | ABOUT HY

First Named Inventor	:	Michael E. Spurlock	
Appln. No.	:	09/928,522	
Filed	:	August 13, 2001	Group Art Unit: 1647
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Docket No.	:	LL31.12-0015	

EXHIBIT G

of

DECLARATION

submitted under 37 C.F.R. 1.132

Information Page Entitled "Sheet for Eppendorf® Sheared Salmon Sperm DNA"
located at http://www.brinkmann.com/product.asp?path=115&ref=136;
obtained from the Internet on November 12, 2004

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SEARCH :

Eppendorf® Sheared Salmon Sperm DNA

All Products ➔ Molecular Technologies ➔ Molecular Biology Reagents ➔ Molecular Biology Reagents Description Group

- Print
- Email This Page

- Manual
- FAQ

sterility tested | exo DNase free | endo DNase free | RNase free | Protease free

Storage at –20 °C

Applications

- Hybridization
- Nucleic acid precipitation

Sheared Salmon Sperm DNA is used as a blocking agent to reduce the background in h experiments.[1] It may be used as a carrier during DNA and RNA precipitation with alcoho preparation is supplied at 10 mg/ml in sterile DNase- and RNase-free Molecular Biology

The Salmon Sperm DNA is sheared by sonication, but it is not fully denatured as supplie Sheared Salmon Sperm DNA for 5 minutes and then quickly cooling it in an ice bath bef recommended. The Sheared Salmon Sperm DNA should be used at a concentration of ! hybridization solutions. The DNA is approx. 200–2,500 bp in length as determined by 1% electrophoresis.

[1] Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989."Molecular Cloning: A Laboratory Manual", 2nd edition. Cold Spring Harbo Spring Harbor, NY. pp 9.8–9.49.

First Named Inventor	:	Michael E. Spurlock	
Appln. No.	:	09/928,522	
Filed	:	August 13, 2001	Group Art Unit: 1647
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Docket No.	:	LL31.12-0015	

EXHIBIT H

of

DECLARATION

submitted under 37 C.F.R. 1.132

Instructions posted at http://www.clarkson.edu/class/by412/word/Northern%20hybridization.doc (obtained from the Internet on November 12, 2004) for Molecular Biology Lab #17 (Northern Transfer and Hybridization Experiment #2: Labeling Probe and Hybridization) taught in Fall, 2004 at Clarkson University in Pottsdam, New York by Craig Woodworth

Molecular Biology Lab 17
Northern Transfer and Hybridization Experiment #2:
Labeling Probe and Hybridization

Background:
The process of labeling and hybridization of Northern blots is performed in a series of steps over several days. The first step is called prehybridization. During this step the membrane containing the RNA is pretreated with a buffer containing blocking reagents such as albumin or salmon sperm DNA. These treatments block any nonspecific 'hot spots' on the membrane that might bind to probe nonspecifically. Often, prehybridization is performed for 30 min using the same buffer that will be used subsequently for hybridization. If prehybridization is inadequate, the blot may have high background with nonspecific probe binding.

Probe labeling is the next step. There are several methods for labeling probes, but all methods require that the DNA molecule first be denatured to separate the 2 complementary strands. This is usually performed by boiling the probe DNA for 5 min then rapidly cooling. Rapid cooling helps to prevent renaturation of the complementary strands.

The most sensitive method of detection uses probes that are labeled with radioactive ^{32}P by random priming or nick translation. These methods add a ^{32}P labeled nucleotide (often deoxy CTP) throughout the probe DNA molecule. The labeling reaction is then passed through a column that binds unincorporated nucleotides and allows the radioactive DNA to elute. This probe is very hot (1×10^8 cpm/μg DNA) and can be used to detect single copy genes with ease. The radioactive DNA that binds specifically to the probe is detected by placing the membrane next to X-ray film, or analyzing the membrane in a phosphoimager. The use of ^{32}P is also associated with potential hazards of external contamination. ^{32}P is very high-energy beta emitter and the researcher must take precautions to shield the body from radiation. Usually this is done by working behind a small Plexiglas shield. All of the experimental waste material must be carefully retained and then disposed of and careful records must be kept. All sources of ^{32}P must be kept under lock and key in the lab (i.e. lab doors are always locked when using ^{32}P) which can be an inconvenience. ^{32}P is rather expensive and has a short half-life. In fact, probes must be used within several days or they decay so much that they are no longer useful.

Another method for labeling probes is by utilizing chemiluminescent detection methods. These are slightly less sensitive than ^{32}P and are not widely used for Southern or Northern blotting when sensitivity is important. However, there is little or no hazard associated with chemiluminscent probes and they have achieved wide use in Western blot applications. This method works by directly labeling the probe DNA with an alkaline phosphatase enzyme. This is achieved by first denaturing the probe DNA and then adding the enzyme along with a cross linking reagent. The alkaline phosphatase labeled DNA that specifically binds to the RNA on the blot is detected by placing the washed blot in a special substrate solution that alkaline phosphatase can dephosphorylate. This reaction is

associated with the release of chemiluminescence which can be detected with X-ray film or a phosphoimager. On the positive side, there is no biological hazard. You can leave the lab doors open, drink coke in the lab, and throw the waste in the regular trash. The probes also have a long half-life and they can be stored for weeks to months.

A third method of detection is colorimetric. This method is very similar to the chemiluminescent protocol, except that the sensitivity is much lower. The probe DNA is labeled with an alkaline phosphatase enzyme by cross-linking and the labeled DNA hybridizes specifically to the complementary RNA on your membrane. The color detection method uses a substrate for the alkaline phosphatase enzyme that becomes insoluble and turns blue when it is cleaved. This method is inherently less sensitive than chemiluminescence or radioactivity. However, it has the advantage that it requires no darkroom or film development reagents.

The third step is hybridization of the labeled probe to the membrane. This is usually performed in a hybridization oven, which carefully regulates temperature and allows the blot to turn constantly so that it is continually bathed in new hybridization solution. There is definitely an optimal temperature for performing hybridization reactions. If the temperature is too low (low stringency) the DNA and RNA strands can join rather easily and they often are able to join even if the complimentary strands don't match completely. For example, at low stringency, a probe for the beta actin gene might cross hybridize with an RNA for alpha actin. The alpha actin RNA from humans might hybridize to the alpha actin probe from a dog. The match doesn't need to be perfect. This can be an advantage if one is searching for RNAs from a new gene family in the same species (maybe you want to use low or relaxed stringency to look for new actin genes). It is also useful if you are searching for an RNA from another species. On the other hand, you can raise the temperature too high (high stringency) so that it is very difficult for any RNA and DNA to hybridize. This is because the temperature is too close to the melting temperature so the hybrids that form are easily denatured again. Under these circumstances, only those sequences that are perfectly matched can form stable hybrids.

How do you choose the right temperature? It depends on many factors including the GC content of the DNA (GC rich DNA melts differently than AT rich DNA), salt concentration (high salt means lower stringency), or the presence of formamide (this lowers the melting temp of DNA). You can also control for stringency of hybridization at the next step, which is washing the unbound probe away from the blot. If you wash at high temperature or low salt concentration, you will remove any hybrids that are not perfectly matched. If you wash at low temperature and/or in high salt, you are leaving many imperfect hybrids on your membrane. Usually, washing proceeds with a low stringency wash at first to remove most of the unbound probe. This is followed by a higher stringency wash. One advantage of using radioactive probes, is that you can easily monitor how 'hot' or radioactive your blot is by simply checking it with the Geiger counter. If it were too 'hot' you would use a more stringent wash. With chemiluminescent or colorimetric detection, you will not know whether you have too much background until you actually develop he blot.

Objectives:
The objective of this lab is to provide experience in labeling DNA with a chemiluminescent probe and using the probe in a Northern hybridization reaction.

Materials:
AlkPhos Direct Labeling and Detection Kit
Hybridization buffer
Primary wash buffer (2M urea, 0.1% SDS, 50 mM sodium phosphate at pH 7, 150 mM Sodium chloride, 1 mM magnesium chloride, 0.2 % blocking reagent)
10X Secondary wash buffer (1M Tris pH 10, 2M sodium chloride)
Alkaline Phosphate Conjugate Substrate Kit
Hybridization oven set at 55°C
Hybridization bottles and nylon mesh
37°C water bath
55°C water bath
Shaker platform
Plastic dishes for washing blots
Pipetters and yellow tips
Ice water bath
Eppendorf microcentrifuge
Previously prepared blot with RNA sample
Eppendorf tubes
Boiling water bath
Dark room with developer and fixer solutions
Film cassettes
X-ray film
Plastic wrap

Methods:

Prehybridization:

1. Preheat the required volume of hybridization buffer to 55°C in the hybridization oven. Heat enough buffer for 0.25 ml/cm^2 of membrane. Also, preheat the glass hybridization bottle containing 15 ml of deionized water.

2. Rehydrate the nylon membrane in water for 5-10 min.

3. Place the blot on a sheet of nylon mesh that is slightly larger (1-2 mm on each side). Make sure that the RNA side of the blot is facing up (check under a UV light if you are not sure). Carefully, roll the blot and mesh and slip the roll into the glass hybridization bottle. The RNA side should face into the hybridization chamber.

4. Place the cap on the tube and hold the tube horizontally. Turn the tube slowly until the membrane unrolls inside the tube and is applied to the walls of the tube.

Inspect the tube and membrane carefully to make sure that there are no air bubbles between the tube wall and the membrane. If air bubbles are present, pull the filter out and start again. Any air bubbles will lead to excess background.

5. Once the filter is applied to the wall of the tube, pour out the water and add about 15 ml of hybridization buffer. Cap the tube and place it into the clips in the hybridization oven. Turn on the speed control such that the bottle turns slowly through the oven. Check to see that the bottle is attached evenly and that the hybridization fluid covers the bottom of the bottle.

6. Allow the blots to prehybridize (before adding the probe) for approximately 30 min. This step is important to block any nonspecific reactive sites on the membrane. Lack of adequate prehybridization can lead to high background due to nonspecific binding of probe to the membrane.

Preparation of probe:

7. Prepare the labeled hybridization probe. Dilute 20 μl of cross linker solution with 80 μl of the water supplied with the kit. This working concentration should be kept on ice.

8. Dilute HPV-16 DNA to a concentration of 10 ng/μl using the water supplied with the kit.

9. Place 10 μl of the diluted DNA sample into an eppendorf tube and denature the DNA by heating for 5 min in a boiling water bath.

10. Immediately cool the DNA on ice for 5 min. Briefly spin the sample in a microcentrifuge to collect the contents at the bottom of the tube.

11. Add 10μl of reaction buffer to the cooled DNA and mix thoroughly but gently. Be sure to keep the tube on ice.

12. Add 2 μl of labeling reagent and mix thoroughly but gently.

13. Add 10 μl of the cross linker working solution. Mix briefly and spin to collect the contents at the bottom of the tube.

14. Incubate the reaction at 37°C for 30 min. The probe can be used immediately or kept on ice for up to 2 hours.

Hybridization reaction:

15. Add labeled probe to the buffer used for prehybridization. Use about 5-10 ng of probe per ml of hybridization buffer. Avoid placing the probe directly on the blot.

Remove a small amount of hybridization fluid from the bottle and mix with the probe before returning this mixture to the bulk of the hybridization buffer.

16. Hybridize at 55°C for 2 days in the hybridization oven.

Post hybridization washes:

17. Preheat the primary wash buffer to 55°C (do not overheat!). Use this in excess at a volume of 2-5 ml per cm^2 of membrane.

18. Carefully remove the roller bottle from the oven. This is easier if you briefly switch off the motor for rotation, and then turn it on again after the bottle is removed. Pour out the hybridization buffer from the roller bottle and add the preheated primary wash buffer (fill the tube halfway).

19. Place the tube with wash buffer back in the hybridization oven and wash for 10 min at 55°C.

20. After 10 min, remove the roller bottle and pour out contents. Add more prewarmed primary hybridization buffer and allow washing in the oven for another 10 min at 55°C.

21. Carefully remove the membrane and mesh from the hybridization bottle using tweezers. Place the membrane in a plastic wash dish with 100-200 ml of secondary wash buffer. Wash with gentle agitation on a shaker platform for 10 min at room temperature. Several blots may be washed in the same secondary wash buffer provided that there is enough volume to allow them to move freely.

22. Pour off the wash buffer and add 100-200 ml of fresh secondary wash buffer. Shake gently for an additional 10 min at room temperature.

Chemiluminescent signal detection:

23. Allow the membrane to drain and briefly dab any excess fluid away with a paper towel

24. Add 3ml of chemiluminescent substrate to your blot and allow it to saturate the membrane. This can be done by placing the blot and substrate in an empty yellow tip box and rocking back and forth.

25. Dab excess substrate from blot using a paper towel and wrap the membrane carefully in plastic wrap. Tape the wrapped membrane to the inside of an X-ray film cassette.

26. Bring the cassette and an unopened X-ray film into the darkroom. Turn on the safelight and shut the door. Check to see that no light is coming in from outside.

27. Open the X-ray film and carefully place a sheet into the cassette so that it covers the blot. Close the cassette cover and allow the film to be exposed for 1 hour. Make sure that the cassette snaps closed (listen for the click).

28. After exposure, return to the darkroom and close the door. Wear latex gloves. Turn on the safelight and open the cassette. Place the film in the developer for approximately 30 sec with periodic agitation. You should see the image of the blot appear.

29. Transfer the developed film to water to remove excess developer and agitate for 2 to 3 min.

30. Place the film in fixer and leave for 2 to 3 min. After this, you can turn on the light. After 30 min, you can place the film in water and wash for 1 to 2 hours. Most modern molecular biology labs have an automated film processor that automatically develops films in 1 to 2 min.

First Named Inventor	:	Michael E. Spurlock	
Appln. No.	:	09/928,522	
Filed	:	August 13, 2001	Group Art Unit: 1647
Title	:	Bovine Leptin Protein, Antisense and Antibody	Examiner: C. J. Saoud
Docket No.	:	LL31.12-0015	

EXHIBIT I

of

DECLARATION

submitted under 37 C.F.R. 1.132

Connolly, Amy L. and Jones, Teri L., Hybridization Buffers I: Comparison of Formamide vs. Aqueous Hybridization Solutions, four pages (KPL Research & Development - August, 2002)

KPL APPLICATION NOTE: August 2002

Hybridization Buffers I: Comparison of Formamide vs. Aqueous Hybridization Solutions

By Amy L. Connolly, M.S., and Teri L. Jones, Ph.D.
KPL Research & Development

KPL offers the flexibility of two types of hybridization buffers for use in Northern and Southern blot detection, one a formamide-based solution and the second an aqueous buffer. Formamide Hybridization Buffer (Cat. No. 50-86-10) has been designed as part of KPL's Non-Radioactive Detector™ Systems to provide optimal performance in nucleic acid blotting applications. A destabilizer, formamide lowers the melting temperature of hybrids thus increasing the stringency of the probe to target binding. Use of this agent with specified hybridization temperatures results in minimal nonspecific hybridization; less optimization of washes is required by the end user. Formamide Hybridization Buffer is a suitable universal hybridization solution.

For those researchers wishing to minimize hazardous waste, the aqueous Membrane Hybridization Buffer (Cat. No. 50-86-08) is a non-hazardous alternative. It is not recommended for all hybridization applications as will be shown in this paper. However, Membrane Hybridization Buffer does perform very well for hybridizations with plasmid DNA and moderately expressed transcripts.

To demonstrate the utility of the Formamide and Membrane Hybridization Buffers, a series of comparative studies were conducted and summarized in this Application Note. Both buffers were tested for relative performance in Northern and Southern blots, detecting plasmid DNA as well as low and abundantly expressed transcripts.

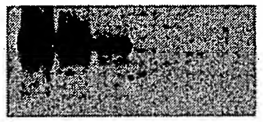
Formamide Hybridization vs. Membrane Hybridization Buffer – Northern Blot Analysis

Using an RNA probe for the detection of a moderate to abundantly expressed message

Two-fold serial dilutions starting at 50 ng of total RNA were electrophoresed on a 1% formaldehyde gel. Using alkaline transfer, the RNA was blotted to Biodyne® B membrane. Hybridization and detection were as follows:

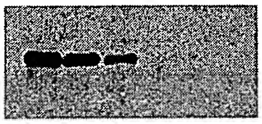
1. The membrane was cut in half to represent two duplicate dilution series. One membrane was pre-hybridized for 1 hour at 65°C in Formamide Hybridization Buffer + 100 ug/mL Herring Sperm DNA. The second membrane was pre-hybridized for 30 minutes at 50°C in Membrane Hybridization Buffer + 200 µg/mL Herring Sperm DNA.
2. A biotinylated 18s riboprobe was denatured at 68°C for 10 minutes and immediately placed on ice. (Note: The RNA probes in these studies were generated via *in vitro* transcription using KPL's Detector RNA *in vitro* Transcription Biotinylation Kit, Cat. No. 60-01-02.)
3. The probe was added to each of the blots at a final concentration of 50 ng/mL of hybridization solution.
4. Blots were hybridized overnight at the same temperature as the pre-hybridization step (65°C in Formamide Hybridization Buffer and 50°C in Membrane Hybridization Buffer, respectively).
5. The blot hybridized in the Formamide Hybridization Buffer was washed 2 x 15 minutes at room temperature in 2X SSPE + 0.1% SDS followed by 2 x 30 minute washes at 65°C in 0.2X SSPE + 0.1% SDS and one final wash for 5 minutes in 5X SSPE.
6. The blot hybridized in the Membrane Hybridization Buffer was washed 2 x 10 minutes at room temperature in 0.5X SSPE followed by 2 x 10 minutes at 55°C.
7. Chemiluminescent detection was performed according to the protocol in the Detector™ AP Chemiluminescent Blotting Kit (Cat. No.'s 54-30-01/02) manual. Exposures of the blots to KODAK BIOMAX Light film were performed at 1 and 10 minutes.

Data and Conclusions



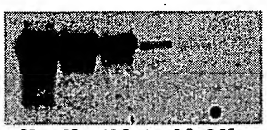
50 25 12.5 1 0.5 0.25

KPL's Formamide Hybridization buffer 1 minute exposure (ng RNA)



50 25 12.5 1 0.5 0.25

KPL's Membrane Hybridization Buffer 1 minute exposure (ng RNA)



50 25 12.5 1 0.5 0.25

KPL's Membrane Hybridization Buffer 10 minute exposure (ng RNA)

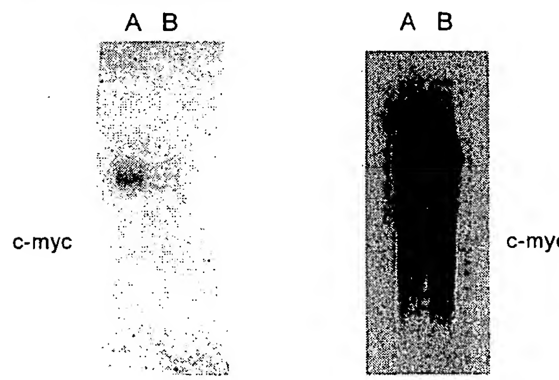
Results of the comparison show that the membranes were sufficiently hybridized such that the riboprobe was able to specifically bind to the target. Additionally, the level of sensitivity achieved is comparable with both hybridization buffers when moderate to abundant messages are probed. The 18s rRNA was detectable to 0.25 ng in both cases. The difference between the blots, however, is observed in the relative sensitivity by exposure time. The Formamide Hybridization Buffer generated stronger signal in a 1-minute exposure to film, whereas equivalent sensitivity was achieved in the blot hybridized with the Membrane Hybridization Buffer after a 10-minute film exposure.

Using an RNA probe for detection of a low expressed transcript

Duplicate lanes of 5 μg of total RNA from WEH1-231 untreated and anti-IgM treated cells were electrophoresed on a 1% formaldehyde gel. RNA was transferred by a 2 hour alkaline method to Biodyne B membrane. Hybridization and detection were as follows:

1. The membrane was cut in half, each blot containing control and treated RNA. One membrane was pre-hybridized for 1 hour in Formamide Hybridization Buffer + 100 μg/mL Herring Sperm DNA at 65°C. The second membrane was pre-hybridized for 30 minutes in Membrane Hybridization Buffer + 200 μg/mL at 55 °C.
2. A biotinylated c-myc riboprobe was denatured at 68°C for 10 minutes and immediately placed on ice.
3. Denatured probe was added to each of the blots to a final concentration of 50 ng/mL of hybridization solution.
4. Blots were hybridized overnight at the same temperature as the pre-hybridization step (68°C in Formamide Hybridization Buffer and 55°C in Membrane Hybridization Buffer, respectively).
5. The blot hybridized in the Formamide Hybridization Buffer was stringently washed 2 x 15 minutes at room temperature in 2X SSPE + 0.5% SDS followed by 2 x 30 minutes at 68°C in 0.2X SSPE + 0.5% SDS and one final wash for 5 minutes in 5X SSPE.
6. The blot hybridized in the Membrane Hybridization Buffer was stringently washed 2 x 10 minutes at room temperature in 0.5X SSPE followed by 2 x 10 minutes at 55°C.
7. Using the Detector™ AP Chemiluminescent Blotting Kit, detection was carried out by standard protocol utilizing 0.5% Detector Block Powder in the block solution. The membrane was exposed to KODAK BIOMAX Light film for 1 minute following a 1 hour incubation.

Data and Conclusions



Formamide **Membrane**

According to the design of this assay, c-myc mRNA should be observed as a single band in the control sample (A) and appear down regulated in the treated sample (B). The blot hybridized with Formamide Hybridization Buffer delivers the expected result. RNA-RNA hybrids are the most stable of the nucleic acid hybrids and thus the most difficult to disassociate even when they are not completely complementary. In this study, Formamide Hybridization Buffer effectively minimized the presence of non-specific hybrids.

However, detection of the transcript with the Membrane Hybridization Buffer could not be achieved.

Significant non-specific binding of the probe to the total RNA was seen when using the Membrane Hybridization Buffer. Multiple attempts were made to increase the stringency of both the hybridization temperature and the washes (data not shown); each blot produced the same pattern. Although the Membrane Hybridization Buffer worked well for the abundantly expressed message, the use of the aqueous Membrane Hybridization Buffer is not recommended in the detection of a low expressed message with a riboprobe. It is also important to note that the stringency of the hybridization temperature and the post-hybridization washes may need to be optimized for each expressed mRNA as detailed in the two experiments shown thus far.

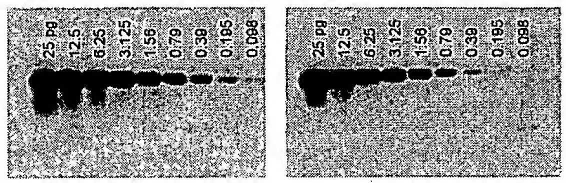
Formamide Hybridization Buffer vs. Membrane Hybridization Buffer – Southern Blot Analysis

Using a random primed DNA probe to detect plasmid DNA

Two-fold serial dilutions of plasmid DNA containing a timp-2 insert were prepared, starting with 25 pg of insert. The dilution series was loaded in duplicate on an agarose gel and subsequently electrophoresed, denatured and neutralized by standard methods. The gel was transferred overnight onto Biodyne® B, a positively charged nylon membrane.

1. The membrane was cut in half to represent two duplicate dilution series. One membrane was pre-hybridized for 1 hour in Formamide Hybridization Buffer + 100 µg/mL Herring Sperm DNA at 42°C. The second membrane was prehybridized for 30 minutes in Membrane Hybridization Buffer + 200 µg/mL at 50°C.
2. A timp-2 random primed biotinylated DNA probe was denatured at 95°C for 10 minutes and immediately placed on ice. (Note: The DNA probe used in this study was biotinylated via KPL's Detector™ Random Primer DNA Biotinylation Kit, Cat. No. 60-01-00.)
3. Denatured probe was added to each of the blots to a final concentration of 50 ng/mL of hybridization solution.
4. Blots were hybridized overnight at the same temperature as the pre-hybridization step (42°C in Formamide Hybridization Buffer and 50°C in Membrane Hybridization Buffer, respecitvely).
5. The blot hybridized with Formamide Hybridization Buffer was stringently washed 2 x 15 minutes at room temperature in 2X SSPE + 0.1% SDS followed by 2 x 30 minutes at 65°C in 0.2X SSPE + 0.1% SDS and one final wash for 5 minutes in 5X SSPE.
6. The blot hybridized in the Membrane Hybridization Buffer was stringently washed 2 x 10 minutes at room temperature in 0.5X SSPE followed by 2 x 10 minutes at 50°C.
7. Detection was carried out by standard protocol using the Detector™ HRP Chemiluminescent Blotting Kit (Cat. No. 54-30-00). Exposure to KODAK BIOMAX Light film was performed for 10 minutes.

Data and Conclusions



Formamide Membrane

Both hybridization buffers worked equally well in the detection of timp-2, each resulting in the detection of as low as 98 fg of plasmid DNA. Relatively, the blot hybridized with the Formamide Hybridization Buffer yielded slightly higher sensitivity with the same exposure time to film. For this type of hybridization where the complexity of the target is not an issue, either buffer is suitable when used under the recommended standard stringent conditions.

However, as the results in the hybridization and detection of the low expressed mRNA show, a similar phenomenon is found in the use of Membrane Hybridization Buffer in single copy genomic Southern blots. As the amount of target in the total sample becomes proportionately smaller, the requirements for stringency becomes greater and the need for further optimization of this hybridization solution also increases. Likewise, greater stringency is required for the hybridization to allow for the specific detection of single copy genes. Therefore, Membrane Hybridization Buffer is not recommended for this application.

Related products:

Description	Size	Catalog No.
Formamide Hybridization Buffer	240 mL	50-86-10
Membrane Hybridization Buffer	240 mL	50-86-08
Detector™ HRP Chemiluminescent Botting Kit	2000 cm^2	54-30-00
Detector™ AP Chemiluminescent Blotting Kit	2000 cm^2	54-30-01
	500 cm^2	54-30-02
Detector™ Random Primer DNA Biotinylation Kit	30 reactions	60-01-00
Detector™ PCR DNA Biotinylation Kit	30 reactions	60-01-01
Detector™ RNA *in vitro* Transcription Biotinylation Kit	20 reactions	60-01-02
GeneRuler™ Biotinylated DNA Ladder	20 - 50 lanes	600-0008
Herring Sperm DNA	40 mg	60-00-14
Biodyne® B Nylon Membrane	20 cm x 1 mL roll	60-00-50
Hybridization Bags, 8" x 10"	50/pk	60-00-51

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Appln. No.	:	09/928,522	
Filed	:	August 13, 2001	Group Art Unit: 1647
Title	:	Bovine Leptin Protein, Antisense and Antibody	Examiner: C. J. Saoud
Docket No.	:	LL31.12-0015	

EXHIBIT R

of

DECLARATION

submitted under 37 C.F.R. 1.132

Brochure entitled Southern (DNA) and Northern (RNA) Hybridizations by GE Osmonics, Inc. (2003)
(Obtained on December 1, 2004 from the Internet at:
http://www.msifilters.com/OsmoLabPage.dll?BuildPage&1&1&921)

Southern (DNA) and Northern (RNA) Hybridizations

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with MAGNA and MagnaGraph Nylon Membranes

Gel Preparation

Southern
Run DNA on an agarose gel with a running buffer of TAE or TBE. Rinse gel with DI water. If necessary, fragment DNA by immersing the gel in 0.25 N HCl for 8-10 minutes. Denature DNA by soaking gel in 1.0 M NaCl/0.5 M NaOH two times for 20 minutes each. Neutralize the gel by soaking in 0.5 M Tris/pH 7.5, 1.5 M NaCl two time for 20 minutes each.

Northern
Run RNA under denaturing conditions in a Glyoxal, Formaldehyde, or Methyl Mercuric Hydroxide gel. Gel should be 0.8-1.5% agarose, 2.5-5.0mm thick. Stain with 33 µg/ml acridine orange in 10 mM $NaPo_4$/pH 6.5, then destain 3 x 15 minutes in buffer. Or, stain with ethidium bromide, 1 µg/ml in 50 mM NaOH for 25 minutes, then destain in 200 mM NaOAc/pH 4.0, 2 x 20 minutes.

Membrane Preparation

Float membrane on distilled water, then immerse until thoroughly wet. Soak the membrane in the transfer buffer until use.

Capillary Transfer

Use a transfer buffer of 10 x SSPE or SSC.

Cut three pieces of filter paper (chromatography grade) three inches longer than the glass plate to be used for the capillary transfer. Saturate the filter paper with transfer buffer and place on top of a glass plate.

Place the gel on top of the filter paper, and the membrane over the gel. Place 5 pieces of filter paper cut to the size of the gel over the assembly. Throughout the transfer, do not allow the paper on top of the gel to contact the paper below the gel. This is done by placing strips of Parafilm around the sides of the gel.

Place the glass plate and the gel assembly on top of a glass baking tray filled with transfer buffer. Allow the bottom layer of filter paper to overhang into the transfer solution in the glass baking tray. Place a 2 inch stack of paper towels on top of the gel assembly and secure it with a light weight. Make sure that the filter paper under the gel is completely saturated. The wicking action of the solution through the gel and up the paper towels allows the solution to transfer the DNA or RNA

molecules to the membrane.

Secure plastic wrap over the entire assembly and place in a cold room for 3 hours to overnight. If the paper towels become saturated with transfer buffer, replace them with dry ones. After transfer, stain the gel with 0.5 µg/ml ethidium bromide to check transfer efficiency.

Alternative Transfer Systems

Vacuum blotting, semi-dry electroblotting, bi-directional transfers, and positive pressure blotting systems can all be used with GE Osmonics nylon membranes. Follow manufacturers instructions, and contact GE Osmonics Technical Services with any questions.

Immobilization

After blotting, wash the membranes in 5 x SSPE at 60°C for 5 minutes. This is an optional step to remove residual agarose. The membrane can be immobilized by baking or UV crosslinking.

To bake the membrane a vacuum oven or convection oven may be used, place the membrane in a 65°-80°C oven for 1 hour or until the membrane is completely dry.

The membrane may be UV crosslinked by exposing the membrane to a controlled UV source. Follow the instructions of the manufacturer of the crosslinker or expose a damp membrane to a transilluminator. The total exposure should be 120mJ/cm. Increased exposure will cause a decrease in signal intensity after reprobing, proportional to the amount of overexposure.

Hybridization Procedure

Hybridization is most commonly done in heat-sealable bags in order to conserve solution and protect researchers from exposure to radioactivity. All hybridization solutions should be filtered before use with a Cameo 25AS, 0.22 µm supported cellulose acetate filter (GE Osmonics catalog #DDA02025S0). (NOTE: Use only cellulose acetate filters; other membrane types may not perform comparably). The low binding Cameo 25AS will filter prehybridization and hybridization solutions without nonspecifically binding essential components of these solutions.

Prehybridization

This step should always be carried out at the temperature of the hybridization. Place the membrane in a heat-sealable bag without the probe in 0.25 ml/cm² of the following prehybridization buffer:

Southern Prehybridization Solution	**Northern Hybridization Solution**
6 x SSPE	5 x SSPE
5 x Denhardt's solution	50% Formamide
0.5-1.0% SDS	0.1-0.5% SDS
50 µg/ml denatured DNA	100 µg/ml denatured DNA
10% Dextran Sulfate	5 x Denhardt's solution
	Shake one to two hours, at 42°C.

Hybridization

Remove prehybridization solution completely from bag, and add the hybridization solution.

Hybridization temperature should be determined by the presence of formamide in the hybridization solution.

Hybridization Temperature Chart

Temperature	% Formamide	Hybridization Solution
42°C	50%	Low Temperature Hybridization Solution
65°C	0%	High Temperature Hybridization Solution

Low Temperature Hybridization Solution

Southern	Northern
50% formamide (47%)	50% formamide (47%)
5 x Denhardt's solution	5 x Denhardt's solution
6 x SSPE	5 x SSPE
0.2% SDS	0.2% SDS
100 µg/ml denatured DNA	100 µg/ml denatured DNA
10% dextran sulfate	10% dextran sulfate

High Temperature Hybridization Solution

Southern	Northern
5 x Denhardt's solution	5 x Denhardt's solution
6 x SSPE	5 x SSPE
0.5% SDS	0.5% SDS
50 µg/ml denatured DNA	100-200 µg/ml denatured DNA
10% dextran sulfate	10% dextran sulfate

NOTE: When using nylon membranes, the potential for backgrounds is greater. Increased volumes of Denhardt's solution and SDS can help further block the membrane.

Dextran sulfate is a rate enhancer for probes larger than 200 base pairs and should not be used with oligonucleotide probes.

Alternative probes (oligonucleotide, RNA, or biotinylated probes) may also be used. Contact GE Osmonics for technical assistance.

Clean probe solutions by adding a small amount of hybridization solution to the probe, and filter it through a Cameo 25AS supported cellulose acetate syringe filter (GE Osmonics catalog #DDA02025S0), to eliminate any contaminants before they come into contact with the transfer membrane.

Denature the probe by boiling in TE buffer for 5 minutes, or incubate with 0.1 volume of 1 N NaOH at 37°C for 5 minutes. Place on ice.

Add the decontaminated probe to the hybridization solution in the heat-sealable bag and reseal. Probe concentration should not exceed 20 ng/ml. Single copy genes or low copy message may require 1-5 10^6 cpm/ml. Probes should be labeled no more than 24 hours before hybridization.

Hybridize 12 hours to overnight. **Important:** If the membrane is to be rehybridized, do not allow it to dry past this point. This will cause irreversible binding to the membrane.

Post Hybridization

Wash temperature should be 25°C below the Tm (melting temperature of the hybrid). If the homology between the probe and membrane-bound DNA is inexact, the wash temperature should be lower.

Stringency Washing Procedure

Low Stringency (for inexact matching)
2 x 15 minutes with 1 x SSC, 0.1% SDS at room temperature
2 x 15 minutes with 1 x SSC, 0.1% SDS at 37°C

Medium Stringency
2 x 15 minutes with 5 x SSC, 0.5% SDS at room temperature
2 x 15 minutes with 1 x SSC, 0.5-1.0% SDS at 37°C
1 x 15 minutes with 0.1 x SSC, 1.0% SDS at 37°C

High Stringency (for perfect hybrids)
2 x 15 minutes with 5 x SSC, 0.5% SDS at room temperature
2 x 15 minutes with 1 x SSC, 0.5-1.0% SDS at 37°C
3 x 15 minutes with 0.1 x SSC, 1.0% SDS at 65°C

NOTE: With nylon membranes, a medium or high stringency washing procedure is recommended to control potential background problems.

Use 0.5 ml of wash solution per square centimeter for all membranes. After washing, remove excess moisture with paper towels. Washes for Northern blots should substitute SSPE for SSC in all wash steps.

Autoradiography

Wrap the membrane in plastic wrap and autoradiograph at -70°C in a cassette with an intensifying screen while slightly damp. Expose the membrane for 25-60 hours.

Probe Removal

Do not allow the membrane to dry if a rehybridization step is intended. Wash in 5 mM Tris-HCl/pH 8.0, 0.2 mM EDTA, 0.05% pyrophosphate, 0.1 Denhardt's for 1-2 hours at 65°C. Rinse in 1 x SSPE. Or, wash in 50% formamide, 6 x SSPE at 65°C for 30 minutes. Rinse in 2 x SSPE.

Please call GE Osmonics' Labstore Help Desk at (877) LAB-STORE (877-522-7867) or by fax (952) 988-6662 for technical support, pricing and availability. For international technical support and distributor locations, call (952) 988-6665.

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Scarpace, P. J., Nicolson, M., and Matheny, M.; UCP2, UCP3 and Leptin Gene Expression: Modulation by Food Restriction and Leptin Journal of Endocrinology 159, 349–357 (1998)

UCP2, UCP3 and leptin gene expression: modulation by food restriction and leptin

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(Requests for offprints should be addressed to P J Scarpace, Geriatric Research, Education and Clinical Center (182), Department of Veterans Affairs Medical Center, Gainesville, Florida 32608–1197, USA)

Abstract

To determine the effects of food restriction and leptin administration on several transcripts involved in energy homeostasis, we examined leptin, uncoupling proteins (UCP) 1, 2 and 3, lipoprotein lipase (LPL), β_3-adrenergic receptors (β_3AR) and hormone-sensitive lipase (HSL) mRNA levels in brown adipose tissue (BAT) and epididymal (EWAT) and perirenal (PWAT) white adipose tissue in three groups of rats. The groups were administered leptin for 1 week, or had food restricted to the amount of food consumed by the leptin-treated animals, or had free access to food. Leptin administration increased serum leptin concentrations 50-fold and decreased food consumption by 43%, whereas serum insulin and corticosterone concentrations were unchanged. Leptin increased LPL mRNA by 80%, UCP1 mRNA twofold, and UCP3 mRNA levels by 62% in BAT, and increased UCP2 mRNA levels twofold in EWAT. In contrast, UCP2 mRNA levels were unchanged in PWAT and BAT. In WAT from food-restricted rats, leptin gene expression was diminished by 40% compared with those fed *ad libitum*. With leptin administration, there was a further 50% decrease in leptin expression. LPL mRNA levels were decreased by food restriction but not by leptin in WAT, whereas β_3AR and HSL mRNA levels were unchanged with either food restriction or leptin treatment. The present study indicates that leptin increases the gene expression of UCP2 in EWAT and that of UCP1, UCP3 and LPL in BAT, whereas reduced food consumption but not leptin, decreases LPL expression in WAT. In addition, with leptin administration there is a decrease in leptin gene expression in WAT, independent of food intake and serum insulin and corticosterone concentrations.

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Introduction

Leptin, the product of the ob gene, is one factor involved in body weight maintenance, and this hormone contributes to the regulation of both food intake and energy expenditure (Campfield *et al.* 1995, Halaas *et al.* 1995, Pelleymounter *et al.* 1995, Scarpace *et al.* 1997). The mechanism of increased energy expenditure appears to involve increased thermogenesis in brown adipose tissue (BAT). We previously reported that leptin administration increases thermogenesis in BAT, including increases in oxygen consumption and uncoupling protein 1 (UCP1) gene expression (Scarpace *et al.* 1997). Thermogenesis in BAT is mediated by sympathetically innervated β_3-adrenergic receptors (Scarpace *et al.* 1992). This process accelerates lipolysis, and the liberated fatty acids serve as substrates for mitochondrial oxidation and provide the signal to activate UCP1. This protein uncouples mitochondria, producing high rates of substrate oxidation and an increase in heat production without the phosphorylation of adenosine 5′-diphosphate (ADP) (Klingenberg 1990). In addition to activating thermogenesis, β_3-adrenergic receptors upregulate the gene expression of UCP1 (Scarpace *et al.* 1994).

Recently, two additional uncoupling proteins, UCP2 and UCP3, have been identified (Boss *et al.* 1997*b*, Fleury *et al.* 1997, Vidal-Puig *et al.* 1997). These uncoupling proteins have 59% and 57% homology, respectively, with UCP1 and 73% homology with each other (Fleury *et al.* 1997, Vidal-Puig *et al.* 1997). In common with UCP1, both UCP2 and UCP3 can partially uncouple mitochondrial respiration (Fleury *et al.* 1997, Gong *et al.* 1997). The expression of UCP2 and UCP3, unlike UCP1, is not limited to BAT. UCP3 is expressed mainly in BAT and skeletal muscle, whereas UCP2 is widely expressed in many tissues, including white adipose tissue (WAT), heart, and muscle in both rodents and humans (Fleury *et al.* 1997, Vidal-Puig *et al.* 1997). In Zucker rats, in which the leptin gene was overexpressed, UCP2 gene expression was increased in WAT (Zhou *et al.* 1997). In

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addition, leptin increases the gene expression of UCP3 in muscle and BAT of ob/ob mice, in which the expression of UCP3 is low (Gong *et al.* 1997). Whether administration of leptin to normal rats upregulates UCP2 and UCP3 gene expression is unknown. We hypothesized that leptin administration, in addition to increasing UCP1 expression, should also increase UCP2 and UCP3 expression.

In addition to mediating thermogenesis in BAT, β_3-adrenergic receptors negatively regulate the gene expression of leptin in WAT (Slieker *et al.* 1996, Li *et al.* 1997). Leptin is primarily synthesized in WAT (Zhang *et al.* 1994, Murakami & Shima 1995, Trayhurn *et al.* 1995*b*). Fasting decreases leptin gene expression, whereas subsequent refeeding increases expression (Li *et al.* 1997, Saladin *et al.* 1995, Trayhurn *et al.* 1995*b*). Insulin and the glucocorticoids also increase leptin gene expression (Slieker *et al.* 1996, De Vos *et al.* 1995, Wabitsch *et al.* 1996) and may mediate the food intake-induced increase in leptin gene expression, whereas catecholamines may mediate the fasting-induced decrease in leptin gene expression. Thus one mechanism by which leptin may negatively regulate its own synthesis is through the inhibition of food intake. Alternatively, leptin may negatively regulate its own synthesis independently of food intake.

To examine the effects of food restriction and leptin administration on several transcripts involved in energy homeostasis, we examined UCP1, UCP2, UCP3, lipoprotein lipase (LPL), and leptin mRNA levels in BAT and in epididymal (EWAT) and perirenal (PWAT) adipose tissue in three groups of rats: administered leptin for 1 week, or having food restricted to the amount of food consumed by the leptin-treated animals, or having free access to food. In addition, mRNA levels for β_3-adrenergic receptors (β_3AR) and hormone-sensitive lipase (HSL) were examined.

Materials and Methods

Animals

Five-month-old male F-344 × Brown Norway rats were obtained from Harlan Sprague–Dawley (Indianapolis, IN, USA). Upon arrival, rats were examined and remained in quarantine for 1 week. Animals were cared for in accordance with the principles of the Guide to the Care and Use of Experimental Animals. Rats were housed individually in microisolated cages with a 12 : 12 h light : darkness cycle (light on from 0700 to 1900 h). Ambient temperature was 26 °C, which is thermoneutrality for these rats (Scarpace *et al.* 1994).

Experimental design

The rats were divided into three groups. The first group was allowed the normal daily consumption of food available *ad libitum* (Purina Rat Chow), and the second group was given 8·5 g food per day, which was the level of food consumption observed after administration of leptin to the rats fed *ad libitum*. The third group was administered mouse leptin (1 mg/day) by osmotic minipumps (model 2ML1, Alzet, Palo Alto, CA, USA), implanted subcutaneously along the back of the rats. Osmotic minipumps containing saline were implanted in the rats fed *ad libitum* and the food-restricted rats. Rats were killed on the 7th day after minipump implantation.

Chemicals

Mouse leptin was supplied by Amgen (Thousand Oaks, CA, USA). All other chemicals were obtained from Sigma Chemical (St Louis, MO, USA).

Tissue harvesting

Rats were killed by cervical dislocation under 85 mg/kg pentobarbital anesthetic. Blood samples were collected by heart puncture and serum harvested by a 30-min centrifugation in serum separator tubes. The circulatory system was perfused with 20 ml cold saline and BAT, epididymal WAT and perirenal WAT excised.

Serum leptin concentrations

Serum leptin concentrations were measured in a solid-phase sandwich enzyme immunoassay (EIA) using a polyclonal rabbit antibody immobilized in microtiter wells (Hotta *et al.* 1996). The antibody was raised against recombinant leptin and was affinity-purified over a recombinant leptin column. Bound leptin was detected with affinity-purified antibody conjugated to horseradish peroxidase and quantitated with a chromogenic substrate (TMB/peroxide). Leptin concentrations were calculated from standard curves generated for each assay using recombinant mouse leptin and then corrected for the crossreactivity with recombinant rat leptin (43%).

Insulin and corticosterone

Insulin concentrations were assayed in a double-antibody reaction using a ruthenylated primary antibody and a biotinylated secondary antibody. The reaction was quantitated by an electrochemiluminescence detection system using the Origen 1·5 analyzer (Igen, Inc., Gaithersburg, MD, USA). Corticosterone was measured by a double-antibody radioimmunoassay (ICN Biomedicals, Irvine, CA, USA).

DNA assay

DNA was determined by fluorescence in delipidated tissue (Labarca & Paigen 1980). WAT (90 mg) was sonicated on

ice for 15 s in 500 μl 2 M NaCl, 1 mM EDTA, 50 mM $NaPO_4$, pH 7·4 buffer. Lipid was removed by the addition of 7 ml acetone (−20 °C), followed by agitation for 10 min and centrifugation at 1800 *g* for 10 min. The pellet was harvested, resuspended in the above buffer and fluorescence was determined at an emission wavelength of 460 nm after the addition of bisbenzimide (1 μg/ml) in a Hoefer (San Francisco, CA, USA) Fluormeter Model TK0100.

Northern analysis and mRNA levels

Total cellular RNA was extracted using a modification of the method of Chomczynski & Sacchi (1987). The integrity of the isolated RNA was verified using agarose gels (1%) stained with ethidium bromide. The RNA was quantified by spectrophotometric absorption at 260 nm using multiple dilutions of each sample.

The probe to detect leptin mRNA was a 33-mer antisense oligonucleotide (5′-GGTCTGAGGCAGGGA GCAGCTCTTGGAGAAGGC) (Trayhurn *et al.* 1995*a*) end-labeled using terminal deoxynucleotidyl transferase (Promega, Madison, WI, USA). The oligonucleotide was based on a region of the mRNA downstream from the site of the primary mutation in ob/ob mice (Trayhurn *et al.* 1995*a*). We previously demonstrated by Northern analysis that this probe binds to a single mRNA species of 4·1 kb (Li *et al.* 1997). The UCP2 cDNA (IMAGE 389584) was provided by Craig Warden (Fleury *et al.* 1997), the UCP3 cDNA was provided by J-P Giacobino (Boss *et al.* 1997*b*), the UCP1 cDNA was provided by Leslie Kozak (Kozak *et al.* 1988), the β_3AR cDNA was provided by James Granneman (Granneman 1991) and mouse LPL and HSL cDNA clones were obtained from the American Tissue Culture Collection (Rockville, MD, USA). The cDNAs were labeled using a random primer kit (Prime-a-Gene, Promega, Madison, WI, USA). For Northern analysis, 20 μg extracted total RNA was fractionated by agarose gel electrophoresis for 2 h. The RNA was then transferred to a charged nylon membrane (Gene Screen, Dupont NEN, Boston, MA, USA) by capillary blotting overnight and fixed by baking at 80 °C for 2 h. For dot blot analysis, several dilutions of the RNA (0·125, 0·25, 0·5, 1·0) were immobilized on nylon membranes using a dot blot apparatus (Biorad, Richmond, CA, USA). The membranes were baked at 80 °C for 2 h. The baked membranes were prehybridized using 25 mM potassium phosphate, 750 mM NaCl, 75 mM sodium citrate, 5 × Denhardt's solution, 50 μg/ml denatured salmon sperm DNA and 50% formamide. After incubation for 14–16 h at 42 °C, the membranes were hybridized with ^{32}P-labeled probes in the prehybridization buffer plus 10% dextran sulfate. After hybridization for 14–16 h at 42 °C, the membranes were washed and exposed to a phosphor imaging screen for 48 h. The latent image was scanned using a Phosphor Imager (Molecular Dynamic, Sunnyvale, CA, USA) and analyzed by Image Quant Software (Molecular Dynamics). Intensities per microgram total cellular RNA were calculated by comparison with internal laboratory standards of WAT or BAT mRNA present on each nylon membrane. To ensure the changes in mRNA levels were specific, nylon membranes probed for leptin mRNA were stripped by brief exposure to boiling water and rehybridized with β-actin. mRNA levels were expressed in arbitrary units (AU), taking values in food-restricted rats as 100 AU.

Table 1 Serum concentrations of leptin, insulin and corticosterone after administration of leptin. Values represent the mean ± S.E. of seven or eight rats

	Leptin (ng/ml)	**Insulin** (ng/ml)	**Corticosterone** (ng/ml)
Treatment			
Food-restricted	4·3 ± 0·5	3·5 ± 0·7	148 ± 18
Leptin	200 ± 65***	2·6 ± 0·5	228 ± 29
Fed *ad libitum*	5·6 ± 0·9	3·4 ± 0·8	184 ± 31

****P*<0·001 compared with other treatments (one-way ANOVA).

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA). When the main effect was significant, the *post-hoc* test was applied to determine individual differences between means.

Results

Leptin administration

Leptin administration for 1 week to rats maintained at thermoneutrality increased serum leptin concentrations nearly 50-fold, whereas serum insulin and corticosterone concentrations were unchanged (Table 1). Leptin treatment resulted in a 43% decrease in food intake (from 16 ± 0·4 g/day to 9 ± 1·1 g/day; P<0·001). The food-restricted rats were limited to a food intake of 8·5 g/day, approximately 50% of the amount of food consumed by the rats fed *ad libitum*. Both food restriction and leptin administration resulted in a significant loss of body weight compared with prerestriction weight (392 ± 11 g compared with 333 ± 9 g; P=0·0023 for food restriction), but there was no greater loss of body weight in the rats administered leptin (388 ± 13 g compared with 341 ± 8 g; P=0·063). In contrast, body weight in the rats fed *ad libitum* increased (from 368 ± 6 g to 376 ± 8 g; P<0·03). With leptin treatment, there was a 36% decrease in PWAT weight and a 22% decrease in EWAT weight

Table 2 White adipose tissue weight, RNA content and DNA content. Values are expressed as total RNA or DNA per fat pad (mean ± S.E. of eight rats)

	PWAT			EWAT		
	Weight (mg)	RNA (μg/pad)	DNA (μg/pad)	Weight (mg)	RNA (μg/pad)	DNA (μg/pad)
Treatment						
Food-restricted	842 ± 69	113 ± 10	205 ± 10	1583 ± 44	365 ± 50	566 ± 85
Leptin	640 ± 55*	105 ± 5	202 ± 17	1241 ± 78**	326 ± 49	409 ± 41
Fed *ad libitum*	1114 ± 104**	136 ± 11	233 ± 22	1966 ± 89**	376 ± 43	542 ± 79

*$P<0.005$ for difference between mean weights by one-way ANOVA, $P<0.05$ for difference with leptin treatment compared with food-restricted rats.
**$P<0.001$ for difference between mean weights by one-way ANOVA, $P<0.05$ for difference from food-restricted rats.

(Table 2). Despite the decrease in PWAT and EWAT weight, total RNA and DNA contents were unchanged with leptin administration (Table 2).

UCP1, UCP2 and UCP3 gene expression

After administration of leptin, UCP2 mRNA levels were examined in BAT, PWAT, and EWAT, whereas UCP1 and UCP3 levels were examined in BAT. Because leptin decreases food intake and any changes in mRNA levels with leptin administration may be secondary to diminished food intake, comparisons were made with rats that had their food restricted to the amount of food consumed by the leptin-treated rats. Northern analysis revealed that the UCP2 probe was bound to a single mRNA species of 1·6 kb in BAT, EWAT, and lung (data not shown), whereas the UCP1 probe was bound to a major band corresponding to 1·5 kb and a minor band at 1·9 kb only in BAT (Scarpace *et al.* 1994). The UCP3 probe bound to a single mRNA species of 2·6 kb (data not shown), similar to that reported by Boss *et al.* (1997*b*). Leptin administration increased UCP2 mRNA levels nearly twofold in EWAT compared with either food-restricted rats or those fed *ad libitum* (Fig. 1), and there was no difference in UCP2 mRNA levels between those two groups of rats (Fig. 1). β-Actin mRNA levels were unchanged with either food restriction or leptin treatment (food restricted, 100 ± 16 AU; leptin, 113 ± 13 AU; *ad libitum*, 111 ± 21 AU). In contrast to values in EWAT, UCP2 mRNA levels were unchanged with leptin treatment in both PWAT and BAT (Table 3). Leptin treatment increased UCP3 mRNA levels 1·6-fold in BAT compared with either food-restricted rats or those fed *ad libitum*, and there were no differences in UCP3 mRNA levels between the latter two groups (Fig. 1). In agreement with our previous findings (Scarpace *et al.* 1997), after leptin administration, UCP1 mRNA levels were increased nearly fourfold in BAT compared with the values in food-restricted rats (100 ± 16 AU compared with 382 ± 53 AU; $P<0.001$). As expected, UCP1 mRNA levels in rats fed *ad libitum* were twofold greater than those in food-restricted rats (100 ± 16 AU compared with 212 ± 33 AU; $P<0.005$). In all experimental conditions, β-actin mRNA in PWAT and BAT was unchanged with leptin administration or food restriction (Table 3).

Leptin mRNA levels

As expected, when food-restricted rats were compared with those fed *ad libitum*, the food restriction alone decreased leptin RNA levels by 40% in both PWAT and EWAT (Fig. 2), whereas β-actin mRNA levels were unchanged by food restriction (Table 3). Despite the decrease in leptin gene expression, there was only a small, non-significant decrease in serum leptin and no differences in either serum insulin or corticosterone concentrations in food-restricted rats compared with the concentrations in rats fed *ad libitum* (Table 1). In addition, with food restriction, there was a 24% non-significant decreases in PWAT weight and a 20% significant decrease in EWAT

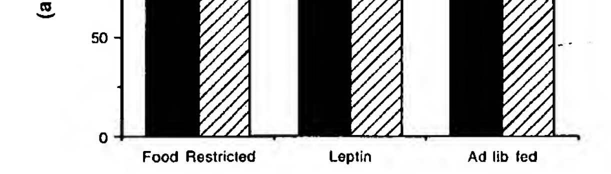

Figure 1 UCP2 mRNA levels in EWAT and UCP3 mRNA levels in BAT from food-restricted rats, leptin-administered rats, and rats fed *ad libitum* (Ad lib fed). Values represent the mean ± S.E. of eight rats and are expressed in arbitrary units, with the values for UCP2 and UCP3 mRNA levels in food restricted animals arbitrarily set at 100 and the S.E. adjusted proportionally. *$P<0.001$ for difference from food-restricted rats or rats fed *ad libitum* (one-way ANOVA).

Table 3 Levels of transcripts in BAT and PWAT after administration of leptin. Values represent the mean ± S.E. of seven or eight rats, and are expressed as arbitrary units, with the level in food-restricted rats set to 100, and the S.E. adjusted proportionally

	BAT (mRNA/μg RNA)			**PWAT** (mRNA/μg RNA)		
	Food-restricted	Leptin	Fed *ad libitum*	Food-restricted	Leptin	Fed *ad libitum*
Transcript						
UCP2	100 ± 4·5	103 ± 9·1	90 ± 5·3	100 ± 7·9	100 ± 11	85·7 ± 5·0
LPL	100 ± 7·5	183 ± 19***	105 ± 10	100 ± 2·9	95 ± 7·1	175 ± 15*
HSL	100 ± 9·9	92 ± 7·0	99 ± 9·1	100 ± 4·5	108 ± 10	103 ± 7·2
β_3AR	100 ± 5·5	97 ± 5·1	93 ± 6·8	100 ± 8·3	95 ± 11	103 ± 11
β-Actin	100 ± 8·1	112 ± 12	113 ± 12	100 ± 6·2	96 ± 9	120 ± 10

*$P<0·05$ compared with food-restricted or leptin-treated rats (one-way ANOVA); ***$P<0·001$ compared with food-restricted rats (one-way ANOVA).

weight, but no changes in DNA or RNA content (Table 2). These data suggest that food restriction diminishes leptin gene expression independently of serum insulin or corticosterone concentrations. After administration of leptin, there was an additional 55% decrease in leptin mRNA levels per microgram RNA in PWAT and a nearly 50% decrease in EWAT compared with levels in food-restricted rats (Fig. 2). Once again, β-actin mRNA was unchanged with leptin administration (Table 3).

When the leptin mRNA data were expressed on a 'per cell' basis – that is, normalized per microgram DNA – there were similar 50% decreases in leptin mRNA levels after administration of leptin (data not shown). The total RNA and DNA contents of PWAT and EWAT were unchanged despite a decrease in tissue weight, suggesting that cell size decreases after both food restriction and leptin administration (Table 2). Cell size may be one factor contributing to the increase in UCP2 mRNA or the decrease in leptin mRNA levels after administration of leptin. However, UCP2 mRNA levels were unchanged with food restriction (Fig. 1), despite the apparent decrease in cell size (Table 2). Moreover, there was a strong correlation between the decrease in leptin mRNA and the increase in UCP2 mRNA levels after administration of leptin (Fig. 3). In contrast, there was no correlation between leptin mRNA and UCP2 mRNA after food restriction (data not shown), the latter of which also decreases cell size (Table 2). These data suggest that the increase in UCP2 mRNA and the decrease in leptin mRNA are more closely associated with leptin administration rather than associated with cell size (Fig. 3).

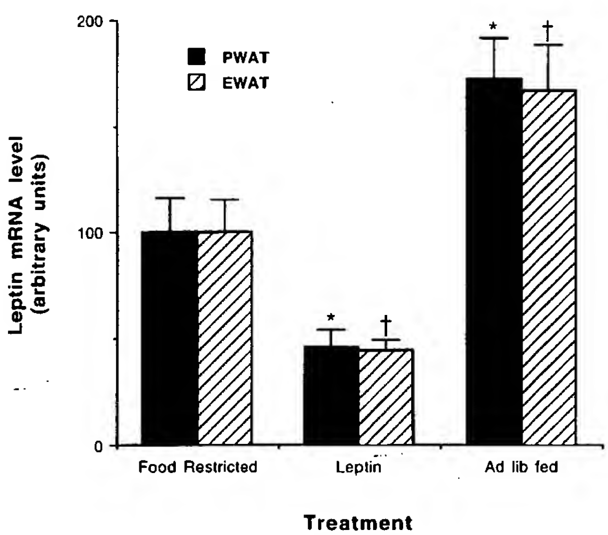

Figure 2 Leptin mRNA levels in PWAT and EWAT in food-restricted rats, leptin-administered rats, and rats fed *ad libitum* (Ad lib fed). Values represent the mean ± S.E. of eight rats and are expressed in arbitrary units, with values for leptin mRNA levels in the food-restricted rats arbitrarily set at 100 and the S.E. adjusted proportionally. *$P<0·001$ for difference with treatment and feeding by one-way ANOVA. $P<0·05$ (leptin) or $P<0·01$ (*ad libitum* fed) for difference from food-restricted rats. †$P<0·001$ for difference with treatment and feeding by one-way ANOVA. $P<0·05$ for difference from food-restricted rats.

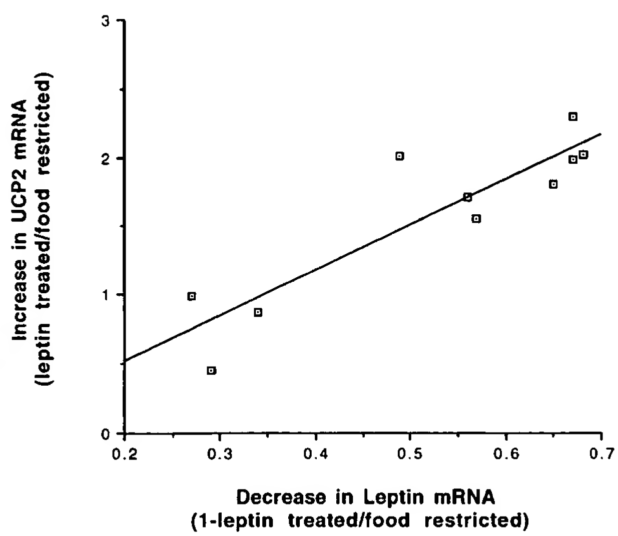

Figure 3 Correlation between the increase in UCP2 mRNA levels after leptin treatment and the decrease in leptin mRNA levels after leptin treatment in EWAT. Each data point represents the ratio of UCP2 or leptin mRNA from an individual rat to the averaged value in the food-restricted rats. Correlation coefficient is 0·81 ($P=0·0004$).

LPL mRNA levels

The enzyme, LPL, is involved in the assimilation of triglycerides into both BAT and WAT, and LPL mRNA levels have been reported to be upregulated by β-adrenergic stimulation in BAT but downregulated in WAT (Auwerx *et al.* 1992, Trayhurn *et al.* 1995*a*). In contrast to its effects on UCP1, food restriction had no effect on LPL mRNA in BAT compared with rats fed *ad libitum* (Table 3). However, leptin administration increased LPL mRNA by 80% in BAT compared with that in food-restricted rats (Table 3). In contrast to the effects on BAT, food restriction diminished LPL mRNA levels in PWAT (Table 3) and EWAT (data not shown) by 40%, but there was no further reduction after administration of leptin. Thus the decrease in LPL mRNA after administration of leptin compared with that in rats fed *ad libitum* appears to be secondary, because of reduced food consumption, rather than a primary effect of leptin.

HSL and β_3AR mRNA

Leptin administration had no effect on two other transcripts involved in energy balance. HSL and β_3AR mRNA levels were unchanged in both BAT (Table 3) and in PWAT (Table 3). In addition, β_3AR mRNA levels were examined in EWAT and were found to be unchanged with leptin administration (data not shown).

Discussion

Leptin contributes to both the negative regulation of food intake and the positive regulation of energy expenditure (Halaas *et al.* 1995, Pelleymounter *et al.* 1995, Scarpace *et al.* 1997). We previously reported that leptin administration increases thermogenesis in BAT, including increases in oxygen consumption and UCP1 gene expression (Scarpace *et al.* 1997). The present study has extended these findings and demonstrated that leptin also increases the expression of UCP2 in EWAT and UCP3 in BAT. UCP2 and UCP3 (homologues of UCP1) are expressed in a variety of tissues and are capable of uncoupling mitochondrial respiration (Fleury *et al.* 1997, Vidal-Puig *et al.* 1997). UCP3, in common with UCP1, is regulated in a manner suggesting a role in thermogenesis and energy balance. In BAT, UCP3 is upregulated by cold, thyroid hormone, and feeding and downregulated by fasting (Gong *et al.* 1997, Larkin *et al.* 1997, Boss *et al.* 1998). In skeletal muscle, UCP3 is upregulated by thyroid hormone but not by cold, and with fasting there is an unexplained increase in UCP3 gene expression (Gong *et al.* 1997, Larkin *et al.* 1997, Boss *et al.* 1998). The biological role of UCP2 is less clear, although some evidence suggests a role for this protein in energy balance and thermogenesis. In UCP1-deficient mice, UCP2 expression in BAT is up regulated, possibly contributing to the surprising absence of obesity in these mice (Enerback *et al.* 1997). In one study, cold exposure upregulated the expression of UCP2 in BAT (Boss *et al.* 1997*a*), but this was not so in another study (Fleury *et al.* 1997). Fasting upregulated the expression of UCP2 in muscle and adipose tissue of humans (Millet *et al.* 1997) and in muscle but not BAT of rats (Boss *et al.* 1997*a*).

Earlier reports suggested a role for leptin in the regulation of UCP2 and UCP3. In rats in which the leptin gene was overexpressed, UCP2 expression was increased in WAT (Zhou *et al.* 1997). Leptin also increased UCP3 expression in ob/ob mice (Gong *et al.* 1997). However, UCP3 mRNA levels were abnormally low in these mice, and the effects of leptin treatment were not investigated in wild-type mice (Gong *et al.* 1997). The present study extends the previous reports by demonstrating that leptin treatment upregulates both UCP2 mRNA in EWAT and UCP3 mRNA in BAT in rodents without any known gene abnormalities in the leptin pathway. These previous reports, coupled with our data, suggest a role for UCP2 and UCP3 as part of the mechanism by which leptin contributes to energy balance. However, in all these studies, the demonstration of an increase in mRNA levels, which could be the result of an increase either in gene transcription or in stability of mRNA, has not been correlated with a functional response, suggesting the roles for UCP2 and UCP3 are not clearly defined. Furthermore, the lack of increase in UCP2 mRNA levels in PWAT suggests that there is a difference in response to leptin depending on the WAT depot. It is known that leptin synthesis is depot-dependent, with subcutaneous fat contributing to serum leptin levels to a greater extent than visceral fat (Takahashi *et al.* 1996).

In addition to UCP1 and UCP2 gene expression, the present report examined the effect of leptin administration on several other transcripts involved in energy balance, including LPL, HSL, β_3AR, and leptin. Leptin increases sympathetic nerve activity and norepinephrine turnover in BAT (Collins *et al.* 1996, Haynes *et al.* 1997). Along with UCP1, LPL is another gene that is believed to be regulated by β-adrenergic stimulation of BAT (Trayhurn *et al.* 1995*a*). This enzyme is involved in the assimilation of triglycerides into both BAT and WAT by hydrolyzing lipoprotein triglycerides before their importation into adipocytes (Auwerx *et al.* 1992). LPL gene expression has a unique pattern of regulation: mRNA levels are upregulated by β-adrenergic stimulation in BAT, but downregulated in WAT (Auwerx *et al.* 1992, Trayhurn *et al.* 1995*a*). The results of the present study indicate that there are parallel increases in UCP1 and LPL mRNA in BAT after administration of leptin compared with levels in food-restricted rats. Food restriction, however, diminished UCP1 mRNA levels, whereas there was no change in LPL mRNA levels in BAT. These data suggest that UCP1

and LPL are regulated differently in BAT, and support our recent study indicating that denervation of BAT prevents the leptin-induced increase in UCP1 mRNA but not the leptin-induced increase in LPL mRNA (Scarpace & Matheny 1998). In contrast to the effects on BAT, we found that administration of leptin reduced LPL expression in WAT, but this reduction was most probably secondary to the leptin-induced decrease in food consumption: that is, leptin had no effect on LPL expression in food-restricted rats. This decrease in LPL mRNA levels in WAT of food-restricted animals may be the physiological response to the diminished demand for lipid assimilation into WAT during the period of food restriction.

Surprisingly, β_3AR mRNA levels were unchanged by leptin in BAT or WAT. We and others have demonstrated that administration of a β_3-adrenergic agonist downregulates β_3AR mRNA in BAT (Granneman & Lahners 1992, Kumar & Scarpace 1998). It is plausible that the putative leptin-induced increase in sympathetic activation of BAT is not sufficient to down-regulate β_3AR mRNA levels, or that down-regulation occurred earlier in the course of the leptin treatment and had adapted by 1 week of treatment.

Leptin gene expression is also inhibited by treatment with a β-adrenergic agonist (Slieker *et al.* 1996, Li *et al.* 1997). In addition, leptin gene expression is stimulated by insulin and glucocorticoids (De Vos *et al.* 1995, Slieker *et al.* 1996, Wabitsch *et al.* 1996, Zheng *et al.* 1996). These hormonal modulators exert their effects directly on WAT and have been demonstrated both *in vivo* after hormone administration (De Vos *et al.* 1995, Zheng *et al.* 1996, Li *et al.* 1997) and *in vitro* in isolated white adipocytes (Slieker *et al.* 1996, Wabitsch *et al.* 1996). Glucocorticoids and insulin may mediate the increase in leptin expression after ingestion of a meal, whereas circulating catecholamines or sympathetic activation may mediate the fasting-induced suppression of leptin gene expression.

In the present study, we demonstrated a suppression of leptin gene expression after administration of leptin. The decrease in food intake with leptin treatment may be contributing to this diminished leptin gene expression. The level of food ingestion may alter serum insulin or corticosterone concentrations, alleviating these stimulatory signals, or it may increase adrenergic stimulation of WAT, activating β_3-adrenergic receptors and the subsequent inhibition of leptin gene expression, or both. We demonstrated that food restriction alone suppressed leptin mRNA levels compared with rats fed *ad libitum*. Moreover, we demonstrated that there was a component of the suppression of leptin gene expression that was independent of food intake. In food-restricted rats, leptin treatment diminished leptin gene expression in both PWAT and EWAT. This decrease in leptin gene expression was not the result of a decrease in either insulin or corticosterone concentrations, as those were unchanged. Thus, in the leptin-treated rats, there was both a food-dependent and a food-independent suppression of leptin mRNA levels.

The decrease in leptin mRNA levels was evident whether expressed per unit RNA, per unit DNA, or per total WAT depot. In addition to the diminished food intake after administration of leptin, there was a decrease in the weight of the PWAT and EWAT depots. However, total RNA and DNA per WAT depot were unchanged, suggesting the loss of weight was due to a decrease in lipid content and, thus, an apparent decrease in cell size.

There are several possible mechanisms that could be contributing to the decrease in leptin mRNA after administration of leptin. First, the decrease in cell size could result in reduced leptin gene expression. However, there was a strong correlation between the increase in UCP2 mRNA and the decrease in leptin mRNA, and UCP2 mRNA was not altered by cell size. For example, food restriction decreased the apparent cell size without a concomitant change in UCP2 mRNA levels in EWAT. Another possibility is that the leptin-induced decrease in leptin expression may be a result of direct feedback through a leptin receptor on adipocytes – leptin downregulated its own expression. The leptin receptor has been identified on adipocytes (Lee *et al.* 1996). Furthermore, the strong correlation between the decrease in leptin mRNA and the increase in UCP2 mRNA suggests that both of these processes are mediated by the same pathway, possibly a leptin receptor. Alternatively, the suppression of leptin gene expression by leptin administration may be indirect, and mediated by some other regulator of leptin gene expression.

In summary, the findings of this study indicate that leptin increases the gene expression of UCP2 in EWAT and UCP3, UCP1 and LPL in BAT, whereas reduced food consumption, but not leptin, decreases LPL expression in WAT. Food restriction diminished UCP1 but not LPL mRNA levels in BAT, suggesting that these transcripts are regulated differently in BAT. In addition, there is a leptin-associated decrease in leptin mRNA levels that is independent of food intake and serum insulin and corticosterone concentrations. Furthermore, these data suggest that the mechanism by which leptin increases energy expenditure is through increased uncoupled respiration both in WAT, involving increased expression of UCP2, and in BAT, involving increased expression of UCP1 and UCP3.

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Growth hormone regulates leptin gene expression in bovine adipose tissue: correlation with adipose IGF-1 expression

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Abstract

Leptin, the product of the *ob* gene, is secreted from white adipocytes and regulates food intake and whole-body energy metabolism. In rodents and humans, leptin gene expression is under complex endocrine and metabolic control, and is strongly influenced by energy balance. Growth hormone (GH) has myriad effects on adipose tissue metabolism. The primary aim of this study was to determine the ability of GH to regulate leptin mRNA expression in bovine adipose tissue *in vitro* and *in vivo*. Incubation of subcutaneous adipose tissue explants for 24 h with GH alone had no effect on bovine leptin gene expression, whereas high concentrations of insulin or dexamethasone (DEX) potently stimulated bovine leptin mRNA abundance. GH, in combination with high concentrations of insulin, DEX, or both, attenuated the ability of insulin or DEX to stimulate leptin expression *in vitro*. These data indicate that GH can indirectly regulate leptin expression *in vitro* by altering the adipose tissue response to insulin or DEX. We extended these studies to examine the ability of GH to regulate leptin expression *in vivo*, using young castrate male cattle treated with no hormone (control) or GH (200 μg/kg body weight per day) for 3 days. GH increased plasma GH and insulin concentrations, but not those of cortisol or non-esterified fatty acid (NEFA) concentrations. GH treatment increased adipose tissue leptin and IGF-1 mRNA concentrations ($n=9$, $P>0{\cdot}001$). In addition, leptin abundance was highly correlated with adipose tissue IGF-1 mRNA in GH-treated animals ($P>0{\cdot}001$). The timing of GH-induced changes in leptin gene expression preceded measurable GH effects on adiposity.

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Introduction

Leptin, the product of the *ob* gene, has been implicated in the regulation of food intake and energy homeostasis in rodents and man. The expression of leptin in adipose tissue is highly correlated with adipose mass and adipocyte size in fully fed individuals (see reviews, Considine & Caro 1997, Houseknecht *et al.* 1998). In addition, leptin gene expression in rodents and humans is under complex endocrine regulation as insulin, glucocorticoids and estrogen have been shown to stimulate leptin expression, whereas leptin concentrations in adipose tissue and serum are down-regulated under conditions such as fasting, adrenergic stimulation, and cold exposure (Considine & Caro 1997, Houseknecht *et al.* 1998).

Growth hormone (GH) plays an important role in the regulation of whole-body energy utilization. GH directly regulates adipocyte metabolism in domestic animals, rodents and man; GH treatment attenuates insulin-stimulated lipogenesis and enhances the adipocyte response to adrenergic stimulation of lipolysis, with the net effect of partitioning nutrients away from the fat cell (see review, Etherton & Bauman 1998). Long-term GH treatment of humans and animals significantly reduces adiposity and increases lean body mass. Furthermore, in growing animals in positive energy balance, exogenous GH treatment causes a reduction in food intake (Etherton & Bauman 1998), suggesting that GH may act on central feeding centers in addition to regulating peripheral tissue metabolism.

Given the profound effects of GH on fat cell metabolism, one might expect that GH would regulate leptin gene expression also. The published data concerning the effect of GH on leptin expression are equivocal. Hardie *et al.* (1996) reported that incubation of isolated rat adipocytes with GH or insulin-like growth factor (IGF)-1 in culture had no effect on leptin synthesis or secretion, whereas others have reported that high concentrations of IGF-1 (100 nM) inhibit dexamethasone (DEX)-induced leptin expression in rat adipose tissue (Reul *et al.* 1997). Several studies (Considine 1997, Matsuoka *et al.* 1999, Miyakawa *et al.* 1998, Rauch *et al.* 1998) have examined

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the effect of long-term administration of GH to GH-deficient patients; however, the results are difficult to interpret, as they are confounded by complex endocrine disorders, GH-induced changes in adiposity, or both. Therefore a primary aim of this work was to determine the effect of short-term GH treatment of healthy, growing animals on leptin gene expression in subcutaneous adipose tissue, before the occurrence of GH-induced changes in adiposity. An additional aim was to determine the effect of GH treatment on insulin and DEX-stimulated leptin expression *in vitro*. We provide the first evidence that GH can regulate leptin gene expression in bovine adipose tissue *in vivo* and *in vitro*, and that mechanisms appear to be independent of adipose tissue mass.

Materials and Methods

Animals and experimental design

All experiments were approved by the Purdue University Committee for Animal Care and Use. Castrate male cattle (Angus, $n=12$, 296·9 ± 27 kg body weight) were used to determine effects of *in vivo* administration of GH on leptin expression. Animals were fed a standard complete diet for growing cattle, which was available *ad libitum*. The diet was formulated from corn, corn silage and protein–mineral supplement to meet National Research Council (1984) requirements for growing cattle (12·5% crude protein, 1·28 mcal net energy gain/kg dry matter). Each animal served as its own control; animals were randomly assigned to Control or GH treatment groups to receive daily injections, respectively, of saline or GH (200 µg/kg body weight per day; Sometribov, Monsanto Corporation, St Louis, MO, USA) for 3 days, following which a subcutaneous adipose tissue sample was obtained by surgical biopsy as described elsewhere (Houseknecht *et al.* 1996). After a 7-day interim period for recovery from previous experimental treatment and biopsy surgery, animals received the alternate treatment and a second adipose tissue biopsy was collected. Adipose tissue samples were snap frozen and stored at − 80 °C until required for analysis for leptin and IGF-1 mRNA abundance. The short duration of GH treatment was chosen to alleviate the confounding effects of GH on adiposity, and we have previously shown that short-term GH treatment (2–3 days) results in significant alterations in the adipose tissue response to hormonal stimulus in cattle (Houseknecht *et al.* 1995, Houseknecht & Bauman 1997). The ability of GH to induce IGF-1 mRNA expression in adipose tissue was used as a positive control, indicating a normal animal response to GH (Coleman *et al.* 1994).

Blood sampling and analyses

Two days before initiation of *in vivo* treatments, a subset of animals ($n=8$) were fitted with indwelling jugular catheters for frequent blood sampling. Plasma samples were collected, beginning 1 h before the third GH injection (or corresponding time during the control period). Samples were collected over 24 h for calculation of mean daily GH concentrations (− 60, − 30, + 10, 20, 30, 40, 50, 60, 80, 100, 120, 150, 180, 240, 300, 360, 480, 600, 720, 840, 1000 and 1440 min relative to injection). Concentrations of GH in plasma were quantified by RIA as previously described (Mollett & Malven 1982). Plasma samples were analyzed by RIA for concentrations of insulin (Linco, St Charles, MO, USA) and cortisol (Coat-A-Count kit, Diagnostic Products, Los Angeles, CA, USA). Concentrations of free fatty acids in plasma were determined using a colorimetric kit (Wako NEFA kit, Biochemical Diagnostics, Edgewood, NY, USA). Inter- and intra-assay coefficients of variation were less than 10% for all assays.

Adipose tissue culture

Subcutaneous adipose tissue (~15 g/animal) was collected from castrate male cattle ($n=11$; 552·4 ± 48 kg body weight; all hormonal treatments were tested within each sample) immediately after exanguination. Adipose tissue explant slices (~100 mg) were prepared using a microtome after dissection to remove connective tissue. Explants were incubated (1 g adipose tissue explants/treatment) in Medium 199 (25 ml/g explants; 5 mM glucose and acetate concentrations; Sigma, St Louis, MO, USA) supplemented with varying concentrations of hormones: basal (no hormone), bovine insulin (1, 10, 100 nM; Sigma), DEX (10, 100 nM; Sigma), GH (4, 40 ng/ml; Monsanto), insulin+DEX (100 nM each), insulin+GH (100 nM insulin, 40 ng/ml GH), GH+DEX (100 nM DEX+40 ng/ml GH), insulin+DEX+GH (100 nM insulin and DEX, 40 ng/ml GH). The concentrations of hormones used span the range reported to be effective in regulating leptin expression in rodent and human adipose tissue (Hardie *et al.* 1996, Kolaczynski *et al.* 1996, Slieker *et al.* 1996). After 24 h in culture (37 °C, 5% CO_2), explants were removed from the media and snap frozen until required for analysis for leptin mRNA abundance. Total RNA was extracted from adipose tissue as described elsewhere (Chomczynski & Sacchi 1987).

Ribonuclease protection analyses

To quantify the abundance of bovine leptin mRNA in subcutaneous adipose tissue, a ribonuclease protection assay (RPA) was developed using a bovine-specific leptin riboprobe as described by Ji *et al.* (1998). Briefly, a 27-bp bacteriophage T7 promoter sequence was added synthetically to the 5′ end of antisense leptin primer (Ji *et al.* 1998). The resulting PCR product yields an antisense RNA when transcribed *in vitro* with T7 RNA polymerase. The transcription reaction was performed with 50 µCi

[α-^{32}P]UTP (800 Ci/mmol) to generate a radiolabeled ribonucleotide probe. The *in vitro* transcription and RPA were accomplished using a commercially available kit (Maxiscript T7+RPA II, Ambion, Austin, TX, USA). Ten micrograms bovine adipose RNA was hybridized to the labeled probe overnight at 45 °C. For quantification purposes, each RNA was co-hybridized to an 18S probe (Ambion). Single-stranded RNA was then digested with a 1 : 50 mixture of RNase I (250 U/ml)–RNase T1 (10 000 U/ml) for 1 h and protected bands were separated by gel electrophoresis (8 M urea, 5% acrylamide). Band intensities were quantified by densitometry or cutting the gel and quantifying radioactivity in gel slices (d.p.m./slice; *in vitro* samples only), or by both techniques. Quantification of radiation in gel slices was highly correlated with densitometry values (data not shown).

Abundance of IGF-1 mRNA in subcutaneous adipose tissue samples was quantified by RPA using an IGF-1 cDNA that represents a 147 bp portion corresponding to exon 3 as described by TaylorRoth *et al.* (1998; a generous gift of Dr Alan Grant, Purdue University). The antisense strand was used to synthesize a labeled riboprobe using T3 RNA polymerase (Promega, Madison, WI, USA). One milligram template was labeled using NEN [α-^{32}P] (800 Ci/mmol). The probe was digested with DNase I for 30 min at 37 °C and gel purified. Twenty micrograms bovine adipose RNA was hybridized to 100 000 c.p.m. of probe at 45 °C overnight. Each sample was also hybridized to an 18S low activity probe to assess uniformity of RNA loading. Non-hybridized RNA was digested with a 1 : 50 mixture of RNase I (250 U/ml)–RNase T1 (10 000 U/ml): buffer (Ambion) for 30 min at 37 °C. The RNA was precipitated, reconstituted in loading buffer and electrophoresed in a denaturing (8 M urea, 5% acrylamide) gel. Band intensity was quantified by densitometry.

Statistical analyses

Data were analyzed by ANOVA using the STATVIEW program for Macintosh (Abicus, CA, USA). When the main effect of treatment was found to be significant (P>0·05), differences among means were determined using Fisher's protected least significant difference *post hoc* test. Data are presented as means ± S.E.M.

Results and Discussion

Endocrine regulation of leptin expression in vitro

To our knowledge, there is currently no published literature describing the direct, endocrine regulation of leptin gene expression in bovine adipose tissue (*in vitro*). Therefore we tested the ability of varying concentrations and combinations of insulin, DEX and GH to regulate leptin expression in tissue culture (Fig. 1). Both insulin and DEX, at high concentrations, significantly stimulated bovine leptin mRNA abundance compared with basal values (P<0·05)which is consistent with several reports in rodents and humans (Hardie *et al.* 1996, Kolaczynski *et al.* 1996, Slieker *et al.* 1996), but conflict with findings of others (Reul *et al.* 1997, Halleux *et al.* 1998). We observed no additive effect of insulin and DEX, similar to the findings of Considine *et al.* (1997), but in contrast to data reported by Hardie *et al.* (1996). Leptin gene expression was not significantly increased by GH treatment alone; however, GH attenuated the stimulatory effects of high concentrations of insulin, DEX, or both. Specifically, leptin mRNA abundance was significantly lower (P<0·03) for 100 nM insulin + 40 ng/ml GH compared with 100 nM insulin. In addition, leptin mRNA abundance was lower (P<0·07) for 100 nM insulin + 100 nM DEX than for 100 nM DEX. Finally, when explants were incubated with high concentrations of GH, insulin and DEX in combination, GH completely attenuated the increase in expression observed with insulin+DEX.

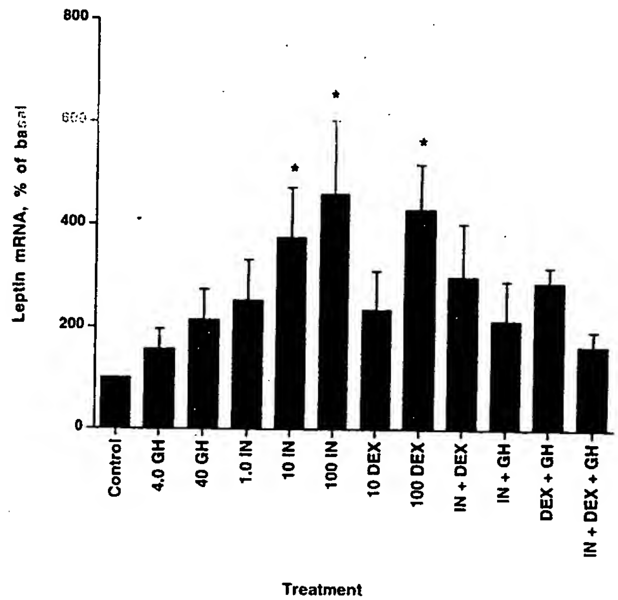

Figure 1 Endocrine regulation of leptin mRNA abundance in bovine subcutaneous adipose tissue explant culture. Subcutaneous adipose tissue was collected at sacrifice from castrate male cattle (n=11; 552·4 ± 48 kg body weight). Explants were prepared from each animal and incubated for 24 hr in Medium 199 in the absence (Control = basal) and presence of insulin (IN), GH or DEX, or combinations thereof: IN (1, 10, 100 nM), DEX (10, 100 nM), GH (4, 40 ng/ml), IN+DEX (100 nM each), IN+GH (100 nM insulin, 40 ng/ml GH), GH+DEX (100 nM DEX+40 ng/ml GH), IN+DEX+GH (100 nM insulin and DEX, 40 ng/ml GH). After incubation, mRNA was prepared from explants and analyzed for leptin mRNA abundance by ribonuclease protection analysis using a bovine-specific leptin probe. Data represent means for 11 animals (tissue from each animal was subjected to all treatments). *P<0·05 compared with Control.

Table 1 Effect of exogenous bovine GH on plasma hormones and metabolites. Values are mean ± S.E.M. for $n=9$ animals

	Control	GH
NEFA (μM)	0·15 ± 0·02	0·20 ± 0·05
Cortisol (ng/ml)	0·35 ± 0·1	0·38 ± 0·05
Insulin (nM)	0·2 ± 0·05	0·5 ± 0·15*

*$P<0·01$ compared with Control.

Effect of exogenous GH administration on plasma GH and IGF-1 mRNA abundance

Next, we chose to expand our findings to examine the effect of bovine GH administration *in vivo* on leptin gene expression. Daily administration of GH significantly ($P<0·001$) increased average daily circulating GH concentrations compared with the control period ($n=9$; control: 2·1 ± 0·6 ng/ml, GH: 62·4 ± 6 ng/ml). Induction of IGF-1 mRNA in adipose tissue in response to GH treatment was used as a positive control, to ensure that animals were eliciting a known biological response to GH treatment. This is especially important, given the short duration of GH treatment, in which no observable change in growth rate or food intake could be quantified. Three of the 12 animals used in this experiment did not acclimatize to the metabolism stalls in which they were housed, as indicated by aggressive behavior during blood sampling, reduced food intake (9·8 ± 1·2 kg dry matter intake/day compared with 13·1 ± 0·7 kg/day for fully acclimatized animals) and lack of body weight gain over the course of the study (both control and GH treatment periods). Consistent with being in zero or negative energy balance, these animals exhibited no increase in IGF-1 expression in response to GH despite significantly increased plasma GH concentrations ($n=3$; 69·1 ± 15·0 ng/ml). Therefore, data from this small subset of animals were removed from the means presented.

In the remaining animals ($n=9$), GH treatment caused a significant ($P<0·001$), 4·8-fold, increase in IGF-1 mRNA abundance in adipose tissue. This is consistent with results previously reported in the pig (Coleman *et al.* 1994) and with increased circulating IGF-1 reported in GH-treated animals (see review, Etherton & Bauman 1998).

Effect of exogenous GH on plasma hormones and metabolites

Exogenous GH treatment caused a twofold increase in plasma insulin concentrations, with no change in plasma cortisol concentrations (Table 1). These data are consistent with previous reports on the effect of GH treatment of cattle (Dunshea *et al.* 1995, Bauman & Vernon 1996). Furthermore, there was no effect of exogenous GH on plasma non-esterified fatty acid concentrations (Table 1).

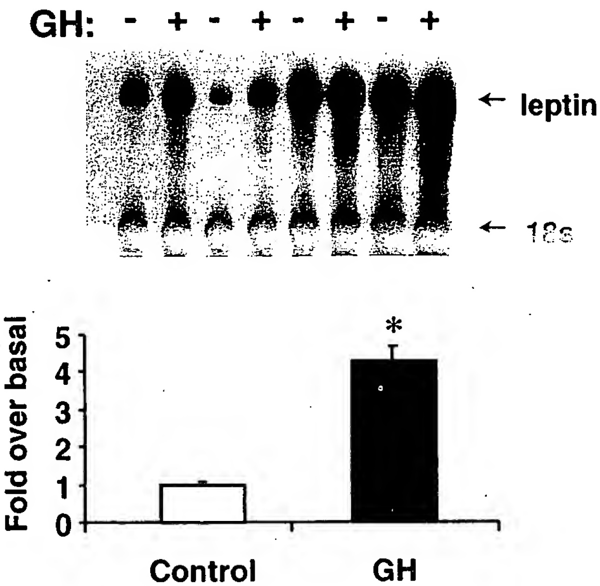

Figure 2 Effect of short-term (3-day) exogenous bovine GH treatment on leptin gene expression in subcutaneous adipose tissue. Castrate male cattle were treated with daily injections of saline (Control) and bovine GH (200 μg/kg body weight per day; Sometribov, Monsanto Corporation) for 3 days (switchback design). Approximately 18 h after the third injection, a subcutaneous adipose tissue biopsy was collected for analysis of leptin gene expression. Total mRNA was extracted from tissues and analyzed for leptin mRNA abundance by ribonuclease protection analysis using a bovine-specific leptin riboprobe. Upper panel: a representative experiment; lower panel: data from all animals ($n=9$). Treatment of animals for 3 days with bovine GH significantly increased leptin mRNA abundance compared with the control period (*$P<0·001$).

Effect of GH on leptin mRNA abundance in subcutaneous adipose tissue

GH treatment significantly ($P<0·001$) increased leptin mRNA abundance in animals exhibiting a positive IGF-1 response (Fig. 2). Interestingly, the three animals in zero/negative energy balance that did not respond to GH with increased IGF-1 exhibited a significant down-regulation of leptin expression in response to GH treatment (~50% reduction; data not shown). Accordingly, concentrations of leptin mRNA were highly correlated with adipose tissue IGF-1 mRNA concentrations during both positive and zero/negative energy balance ($n=12$, $r=0·81$, $P<0·001$; Fig. 3).

General Discussion

We have provided evidence that GH can regulate leptin gene expression independently of GH-induced effects on adiposity. We tested this hypothesis using both *in vitro* and *in vivo* approaches, and provide the first evidence of GH

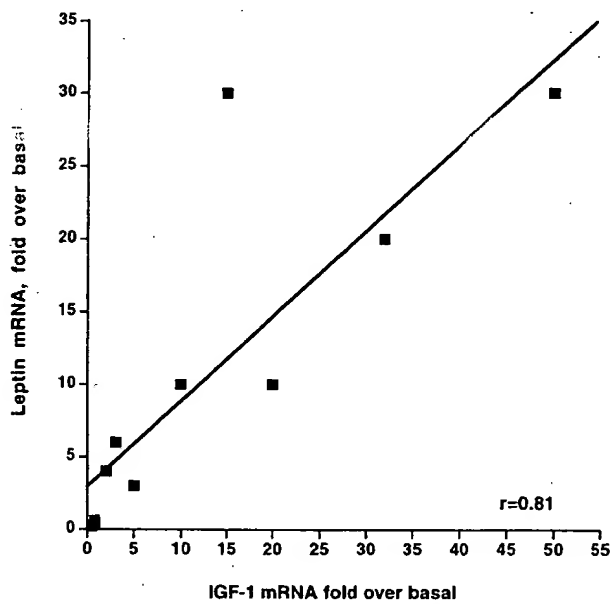

Figure 3 Correlation between expression of IGF-1 and leptin in subcutaneous adipose tissue. Castrate male cattle (n=12) were treated with daily injections of saline (Control) and bovine GH (200 µg/kg body weight per day; Sometribov, Monsanto Corporation) for 3 days (switchback design). Approximately 18 h after the third injection, a subcutaneous adipose tissue biopsy was collected for analysis of leptin and IGF-1 gene expression by ribonuclease protection analysis. Leptin expression was highly correlated (r=0·81, P<0·001) with IGF-1 expression in adipose tissue.

regulation of leptin gene expression in cattle. It has been well documented in studies with rodents and humans that insulin and glucocorticoids are potent stimulators of leptin gene expression (see reviews, Considine & Caro 1997, Houseknecht *et al.* 1998). Recently, Isozaki *et al.* (1999) have reported that GH administration (7 days) to obese, hyperleptinemic Zucker fatty rats reduced leptin mRNA expression in visceral, but not subcutaneous, adipose tissue. However, there is a paucity of data that indicate that GH regulates leptin expression in adipose tissue of normal animals, independent of GH-induced changes in adiposity.

Work by Hardie *et al.* (1996) showed that, when isolated rat adipocytes were incubated with either GH or IGF-1 alone, there was no effect on leptin secretion into the incubation medium, in contrast to increased secretion in response to insulin or glucocorticoids. We extended the work of Hardie *et al.* (1996) to include incubation of bovine adipose tissue explants with insulin, DEX, and GH alone and in combination, and confirmed that GH alone had no effect on leptin gene expression in bovine subcutaneous adipose tissue. However, when incubated in combination, GH attenuated the insulin- or DEX-induced stimulation of leptin gene expression. These *in vitro* data are consistent with work of others (see reviews, Bauman & Vernon 1993, Etherton & Bauman 1998) that has shown that one effect of exogenous GH treatment is to inhibit insulin-mediated events in the adipocyte, such as stimulation of lipogenesis, inhibition of lipolysis and activation of adipogenic genes.

To examine further the role of GH in regulating leptin expression in adipose tissue, and to delineate endocrine mechanisms underlying leptin gene expression in cattle, we extended our work to include short-term exogenous GH treatment *in vivo*. Much of the literature in which the effects of exogenous GH of human subjects on leptin gene expression has been examined is difficult to interpret, because of the confounding effects of GH deficiency or chronic disease on leptin expression, in addition to GH-induced changes in adiposity. We hoped to minimize such confounding effects in our study by choosing a short duration of treatment (3 days) and treating normal animals that had no GH-related pathology. We were fairly confident that this short window of treatment should be sufficient to reveal the effects (if any) of GH on adipose tissue leptin expression, as we and others (Houseknecht *et al.* 1995, Kerber *et al.* 1998) have shown that GH-induced changes in adipocyte biology, such as an increased response to β-adrenergic agents and a reduced response to insulin, are in place within 15–24 h of treatment. Indeed, after 3 days of GH treatment, there was a significant up-regulation of adipose tissue IGF-1 mRNA, consistent with an appropriate biological response to GH (Coleman *et al.* 1994). Furthermore, the GH-induced up-regulation of IGF-1 mRNA in adipose tissue was accompanied by a significant increase in leptin mRNA abundance.

The differing effects of GH on bovine leptin gene expression we report in the *in vitro* versus *in vivo* studies highlights the complexity of the *in vivo* system. The simplified *in vitro* system provides evidence that GH can modulate leptin expression, presumably via alteration in tissue response to hormones such as insulin and DEX. *In vivo*, the hormonal and nutritional milieu are more complex and, as GH inhibited leptin gene expression *in vivo*, it is tempting to speculate that GH effects are modulated by other hormonal and nutritional factors. The fact that others (Hardie *et al.* 1996, Isozaki *et al.* 1999) have shown no direct effect of IGF-1 on leptin gene expression supports the notion that GH effects on leptin expression are not mediated by IGF-1. However, others (Boni-Schnetzler *et al.* 1999, Reul *et al.* 1997) have reported a negative effect of IGF-1 on leptin gene expression, thus more research is needed to clarify if the effects of GH on leptin gene expression are mediated via effects on IGF-1.

The high correlation between IGF-1 expression and leptin expression in adipose tissue was quite striking. Coleman *et al.* (1994) previously reported that GH potently stimulates IGF-1 gene expression in adipose tissue of pigs; to our knowledge, our data are the first such report in cattle. Furthermore, it was interesting that, in the three

animals that were in zero/negative energy balance during both the control and GH treatments, there was a significant down-regulation in leptin expression and a down-regulation or no change in IGF-1 expression in response to GH (Fig. 3). Thus the strong correlation between leptin and IGF-1 mRNA abundance holds for all animals tested, regardless of response to GH. Similarly, Fine *et al.* (1998) have recently reported a high correlation between adipose tissue leptin and IGF-1 expression in fed and fasted rats. However, although leptin and IGF-1 mRNA expression are correlated in adipose tissue of rodents and cattle, these data do not prove that leptin gene expression is regulated by IGF-1.

The reciprocal effects of GH to stimulate or inhibit leptin gene expression, depending upon the animal's energy balance, are consistent with the feeding behavior commonly observed with GH treatment. When growing animals in positive energy balance are treated with exogenous GH, there is a characteristic decrease in food intake (see review, Etherton & Bauman 1998). As experimentally induced increases in leptin have been shown to inhibit food intake in rodents (see reviews, Considine & Caro 1997, Houseknecht *et al.* 1998), it is tempting to speculate that a GH-induced increase in leptin expression could provide such a signal to reduce food intake in cattle. Conversely, when GH is administered to animals in negative energy balance, there is typically an increase in food intake to support the increased metabolic demand incurred by GH treatment (Etherton & Bauman 1998). Again, on the basis of rodent data, a GH-induced decrease in leptin expression could provide a profound stimulus to eat, under these conditions.

Finally, several groups have reported that leptin regulates GH secretion in rodents (Carro *et al.* 1997, 1998, 1999, Tannenbaum *et al.* 1998, Vuagnat *et al.* 1998), sheep (Roh *et al.* 1998) and pigs (Barb *et al.* 1998), and that exogenous leptin is able to prevent the fasting-induced decrease in GH secretion, but not IGF-1 secretion, in fasting rats (LaPaglia *et al.* 1998). Our data coupled with data on the regulation of GH secretion by leptin, highlight the important link between leptin and the GH–IGF-1 axis in communicating the nutritional and metabolic status of the animal, and have important implications in the regulation of animal growth and development.

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Regulation of leptin gene expression by insulin and growth hormone in mouse adipocytes

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Abbreviations: DEPC, diethyl pyrocarbonate; BW, body weight; SDS, sodium dodecyl sulfate; ECL, enhanced chemiluminescence; IgG, immunoglobulin G; ELISA, enzyme-linked immunosorbent assay; s.c, subcutaneous

Abstract

The role of leptin in the control of obesity, insulin resistance and type II diabetes has been reported, however, the regulatory mechanism of leptin in animals affected by hormones is not clearly understood. In this study, the effects of insulin, epinephrine, growth hormone or dexamethasone on the expression of leptin was examined in mouse primary adipocytes. The leptin expression was also studied in the adipose tissue of the mouse treated with insulin or growth hormone (0.3 or 0.6 units/animal). Insulin (100 nM) or dexamethasone (100 nM) stimulated leptin mRNA transcription while epinephrine (100 nM) alleviated its transcription in mouse primary adipocytes. The level of leptin protein in cultured media of adipocytes treated with insulin or dexamethasone was higher than that of the control group but growth hormone or epinephrine treatment had no effect on them. Insulin administration (0.6 units/mouse) enhanced leptin mRNA as well as leptin protein in mouse adipose tissue but growth hormone administration (0.3 or 0.6 units/mouse) had no effect on them. Leptin protein level in sera of mice injected with insulin or growth hormone was not significantly different from that of control group. These results indicate that both insulin and dexamethasone stimulate leptin gene expression and secretion of its product, whereas, growth hormone has no effect on the expression of leptin gene in mouse adipocytes.

Keywords: Leptin, Insulin, Growth hormone, Expression

Introduction

Leptin, a cytokine secreted mainly from adipocytes is *ob* gene product of which primary function is thought to regulate energy balance (Zhang *et al.*, 1994). It is well known that leptin has physiological effects such as the reduction of food intake (Pelleymounter *et al.*, 1995) and increase of metabolic rate by signal transduction between brain and adipose tissue (Campfield *et al.*, 1995). Recently, the potential role of leptin in cell proliferation and angiogenesis has also been suggested (Park *et al.*, 2001). The discovery of anti-obesity action of leptin contributed to the understanding of clinical-pathology related to the change of body weight and body composition. It has been documented that obesity is associated with insulin resistance and non-insulin dependent dialetes mellitus (Saladin *et al.*, 1995). But the role of leptin in the control of insulin resistance and diabetes melitus is unknown.

The stimulatory effect of insulin, cAMP and glucocorticoid on the gene expression and secretion of leptin has also been reported (Slieker *et al.*, 1996). On the other hand, leptin inhibited insulin secretion either by the activation of K_{ATP} chanel (Felber *et al.*, 1993) or by the feedback inhibition of insulin gene expression (Docherty *et al.*, 1991), suggesting that insulin and steroid hormones are involved in the regulation of leptin status in animals.

Reduced secretion and insensitivity of growth hormone are seen in obese patients (Rodbell, 1964; Bercu *et al.*, 1998), and the secretion of growth hormone is influenced by metabolic regulators such as glucose, free fatty acids, amino acids, steroid hormones and thyroid hormones (Stewart *et al.*, 1989; Lanzi *et al.*, 1997; Alba-Roth *et al.*, 1998). But, the functional relationship between leptin and growth hormone has not yet been determined.

These results implicate that leptin involves in signal transduction system related to body fat accumulation and various hormones related to body fat composition might influence its function. The metabolic state of animal affected by hormones and various metabolic mediators is closely linked to obesity and expression of *ob* gene. In this study, obesity of animals under the influence of hormones regulating the metabolic state of animal was evaluated *in vitro* by measuring the expression of leptin in the adipocytes of mouse treated with insulin, epinephrine, growth hormone or dexamethasone. In addi-

tion, effects of insulin and growth hormone on the leptin gene expression in mouse adipose tissue were investigated.

Materials and Methods

Animals and Cells

Fifteen male ICR mice (~50 g BW) were fed with commercial rat chow *ad libitum* and tap water was supplied to consume freely. Animals were divided into five groups (3 mice per group) and 0.3 or 0.6 units of insulin or growth hormone (dissolved in 0.5 ml of physiological saline) per mouse were injected s.c. Control group received the same amount of physiological saline solution. Blood was drawn from each mouse at 24 h after the injection of hormone and serum was separated from blood by centrifugation at 1,000 *g* for 15 min at room temperature and saved for the analysis. All mice were sacrificed after drawing blood. Adipose tissue in epididymal fat was removed and washed twice with saline solution and saved for the analysis of leptin expression.

For the preparation of mouse adipocytes, adipose tissue was sliced into 3 mm thickness, added with 5 ml of bicarbonate buffer (25 mM $NaHCO_3$, 12 mM KH_2PO_4, 1.2 mM $MgSO_4$, 4.8 mM KCl, 120 mM NaCl, 1.4 mM $CaCl_2$, 5 mM glucose, 2.5% BSA, 50 units penicilline, 10 ng streptomycin, pH 7.4) containing 10 mg of bacterial collagenase and digested for 1 h at 37°C with a mild rocking. Adipocytes released from the tissue were harvested by centrifugation (400 *g*, 15 min). Cells in upper phase in centrifuge tube were collected, washed three times with the fresh bicarbonate buffer and resuspended in Dulbecco's modified Eagles medium (DMEM) containing 10% fetal calf serum. For the treatment of hormones adipocytes were divided into five groups; insulin, epinephrine, growth hormone, dexamethasone and control groups. Cells in culture plate (7.5×10^5 cells/ml) for each group were treated by addition of 100 nmol of each corresponding hormone to DMEM (4 ml/well) and incubated for the experimental period indicated. Cells and media in each plate were harvasted at 0, 6, 12 and 24 h after the hormone treatment and saved for the analysis of leptin. Preparation and culture of adipocytes were performed in aseptic conditions described by Rodbell (1964).

Quantitative RT-PCR

Total RNA in mouse adipose tissue was isolated using Tri agent (Amersham Life Science Ltd., Buckinghamshire, England). The detailed methods are described in the manual supplied by the company. Reverse transcription of leptin mRNA was carried out by incubating a mixture (final 20 μl) containing 1 μg of total RNA dissolved in 8 μl of DEPC-treated water with 2 μl of 5 × reaction buffer (250 mM Tris-Cl, pH 8.3, 25 mM KCl, 50 mM DTT, 50 mM $MgCl_2$) at 65°C for 10 min, followed by incubation for 1 h at 37°C after addition of 1 μl of antisense primer for leptin cDNA (100 pmol), 1 μl of dNTP (10 mM), 1 μl of RNase inhibitor (20 units), and 1 μl of reverse transcriptase (10 units). After the incubation, the reaction mixture was taken into boiling water bath and incubated for 5 min to inactivate the enzyme. Quantitative PCR was performed by the method of Saiki *et al.* (1988) using sense and antisense primers corresponding to leptin cDNA sequence together with leptin cDNA as template.

Northern blot hybridization

Total RNAs isolated from adipocytes or adipose tissue of mice administered with various hormones were subjected to formaldehyde agarose gel electrophoresis according to the previously described method (Kim *et al.*, 2000) and RNAs separated on the gel were electrophoretically transfered onto nitrocellulose membrane. Leptin mRNA was hybridized with ^{32}P-labeled leptin cDNA and detected by autoradiography.

Western blot immunodetection

Proteins from adipocytes and adipose tissue of mice treated with various hormones were extracted by differential centrifugation after homogenizing cells or tissue with tissue homogenizer, and soluble fractions were subjected to SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (1970) and proteins separated on the gel were electrophoretically transfered onto nitrocellulose membrane. Leptin protein was identified by ECL-associated immunochemical analysis using anti-leptin rabbit antibody as a primary antibody (1:2,000 dilution) and horse radish peroxidase-conjugated anti-rabbit IgG antibody as a secondary antibody (1:1,000 dilution). The content of leptin in sera of mice was measured by two-site ELISA according to the method of Johnston and Thorpe (1987). The antibodies used in ELISA were same as that used in Western blot immunodetection analysis. Total protein content was measured by Bradford method (1976).

Results

Effect of hormones on the transcription of leptin mRNA in mouse adipocytes

Adipocytes treated with insulin (100 nM) caused an increase of the leptin mRNA expression level, whereas the epinephrine (100 nM) affected the cells by lowering leptin expression. Both growth hormone and dexamethasone also induced an elevation of leptin mRNA level in mouse adipocytes as shown in RT-PCR analysis (Figure 1).

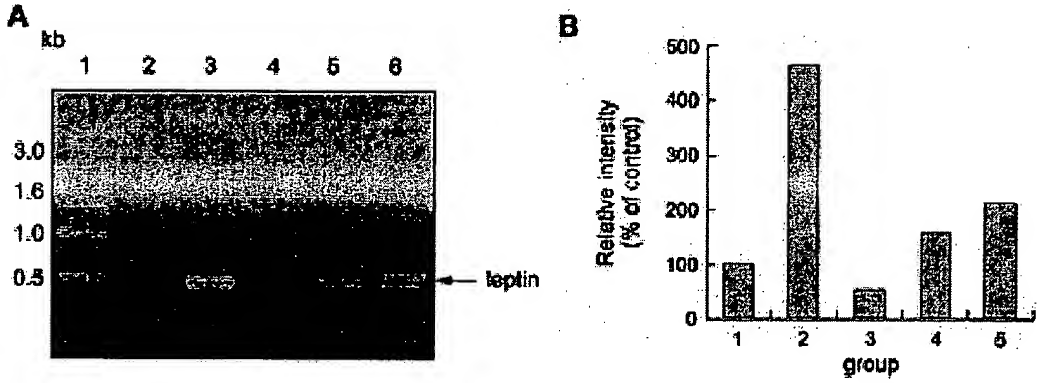

Figure 1. Effect of insulin, epinephrine, growth hormone and dexamethasone on the expression of leptin mRNA in mouse adipocytes. Mouse adipocytes were treated with each respective hormones (100 nM) and cultured for 24 h. Cells were harvested and total RNAs were prepared and used as templates for the detection of leptin mRNA by RT-PCR (A). Lane 1, DNA molecular size marker; lane 2, control; lane 3, insulin treated; lane 4, epinephrine treated; lane 5, growth hormone treated; lane 6, dexamethasone treated. Relative intensity of the amplified cDNA bands of leptin (B) was calculated from densitogram of the cDNA bands appreared in A panel and expressed as % of the control. Group 1, control; group 2, insulin treated; group 3, epinephrine treated; group 4, growth hormone trearted; group 5, dexamethasone treated.

Leptin protein level in the culture media of mouse adipocytes treated with various hormones

Treatments with insulin (100 nM) or dexamethasone (100 nM) of mouse primary adipocytes increased leptin protein level in the culturerd media of mouse adipocytes, indicating that both insulin and dexamethasone stimulated the expression of leptin gene and secretion of its product in adipocytes. However, treatments with neither growth hormone (100 nM) nor epinephrine (100 nM) caused any significant change in leptin protein level in the cultured media of adipocytes as compared to that of the control group, indicating that both growth hormone and epinephrine had no effect on the expression of leptin gene and secretion of its product in adipocytes (Figure 2). Leptin protein level in adipocytes gradually increased for the first 12 h after the insulin treatment, then it decreased at 24 h after the hormone treatment, indicating that the effect of insulin added to the culture media of adipocytes may not persist longer than 12 h.

Effect of hormones on leptin protein level in mouse adipocytes

Mouse primary adipocytes treated with insulin (100 nM) increased cellular leptin protein level, but the treatment with epinephrine rather decreased it. Administration of growth hormone or dexamethasone caused a slight increase in the leptin protein level in adipocytes without any significance (Figure 3). Leptin protein level in mouse

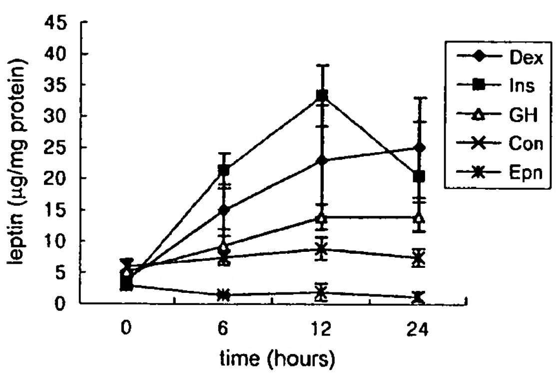

Figure 2. Expression and secretion of leptin from mouse adipocytes treated with 100 nM of each insulin (Ins), epinephrine (Epn), growth hormone (GH) and dexamethasone (Dex). Control (Con) cells were treated with saline and cultured for 24 h. A fraction of the culture media (1 ml) were taken at 0, 6, 12, 24 h after the hormone treatment and leptin protein secreted into media of each time point was measured by ELISA method. Leptin content was calculated from standard curve constructed from purified recombinant leptin.

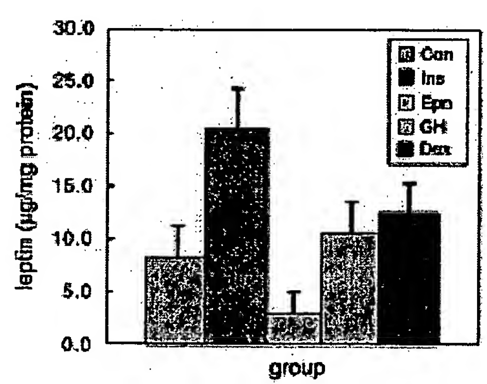

Figure 3. Detection of leptin protein in cultured mouse adipocytes treated with 100 nM of insulin (Ins), epinephrine (Epn), growth hormone (GH) and dexamethasone (Dex) and cultured for 24 h. Control cells (Con) received no hormone. Leptin protein in cell extract was subjected to ELISA. The amount of leptin was calculated from standard curve constructed by reference leptin protein (recombinant leptin). Bars indicate S.D.

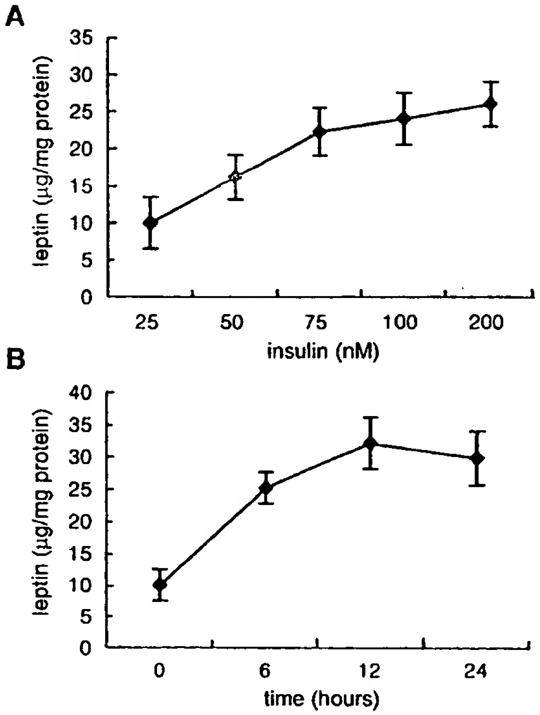

Figure 4. Effect of insulin on the leptin expression in cultured mouse adipocytes. Mouse adipocytes were treated with various amounts of insulin (25, 50, 75 100, 200 nM) and cultured for 24 h (A). Adipocytes treated with insulin (100 nM) were cultured for 24 h and harvested at various time points indicated (B). Leptin in the cell extracts was measured by ELISA method.

adipocytes increased by insulin treatment in a dose-dependent manner up to 75 nM of insulin in the media but its further increment had no additional effect on leptin protein level in adipocytes (Figure 4A). Treatment of insulin (100 nM) to mouse adipocytes gradually increased leptin protein level in the cells for the first 12 h of culture, whereas it decreased slightly at 24 h of culture (Figure 4B).

Effect of insulin and growth hormone on the expression of leptin in mouse adipose tissue

The level of leptin mRNA in adipose tissue was increased by insulin (0.6 units/mouse) administration but the administration of growth hormone (0.3 or 6 units/ mouse) had no effect on mRNA transcription of leptin gene with (Figure 5). Western blot immuodetection for leptin protein expressed in adipose tissue of mice administered with insulin or growth hormone revealed that insulin increased leptin protein level in adipose tissue but growth hormone had no significant effect on it (Figure 6). Insulin administration to mice showed no change in leptin protein level in sera of mice but growth hormone administration rather decreased it with no

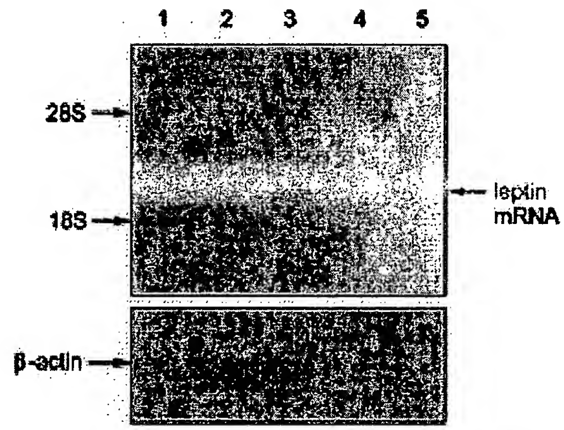

Figure 5. Effect of insulin and growth hormone on the expression of leptin mRNA in mouse adipose tissue. Mice were administered s. c. with insulin (0.3 or 0.6 units per mouse) or growth hormone (0.3 or 0.6 units per mouse) and sacrificed after 24 h. Total RNA was prepared from adipose tissue (epididymal fat) and was subjected to Northern blot hybridization using a ^{32}P-labelled leptin cDNA probe. Lane 1, control (mice injected with saline); lane 2, mice injected with insulin (0.3 units); lane 3, mice injected with insulin (0.6 units); lane 4. mice injected with growth hormone (0.3 units); lane 5, mice injected with growth hormone (0.6 units).

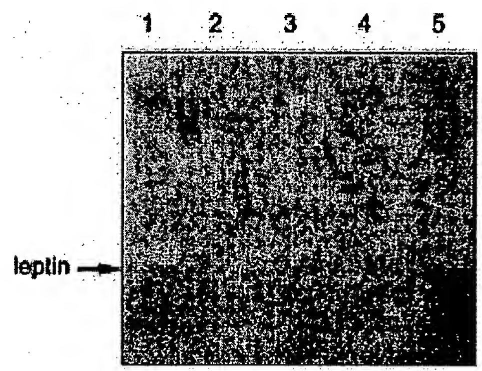

Figure 6. Western blot immunodetection of leptin protein in adipose tissue of mice administered with insulin or growth hormone. Lane 1, control mice injected with saline; lane 2, mice injected with insulin (0.3 units per mouse); lane 3, mice injected with insulin (0.6 units per mouse); lane 4, mice injected with growth hormone (0.3 units per mouse); lane 5, mice injected with growth hormone (0.6 units per mouse).

statistical significance (Table 1).

Discussion

Leptin is synthesized in adipocytes and secreted into ciculateing blood to be transported to leptin receptors present at hypothalamus where it regulates food intake, thermogenesis and energy metabolism in adipose tissue (Bai *et al.*, 1996). But the presence of leptin receptors in

Table 1. Serum leptin level of mice administered with insulin or growth hormone

Group	leptin (ng/ml)			means
Control	10.8	12.4	9.9	11.0±1.3
Insulin (0.3U)	10.2	13.1	16.3	13.2±3.0
Insulin (0.6U)	13.0	14.6	11.5	13.0±1.6
Growth hormone (0.3U)	8.9	9.7	9.6	9.3±0.6
Growth hormone (0.6U)	9.2	10.3	9.4	9.6±0.7

Leptin contents in sera were determined by ELISA assay. Each hormone was administered s.c. into mouse once a day for 3 days and serum sample was taken at 12 h after the last administration. No significant differences between groups are observed.

various peripheral tissues has been reported (Lee *et al.*, 1996).

The increased leptin level in the media of adipocytes treated with insulin or dexamethasone in the present study reflects a consequence of an increased expression of leptin gene by these hormones in adipocytes. This result is consistent with the previously reported results by other investigators (Muller *et al.*, 1997; Fain *et al.* 1998). From these results, it is assumed that insulin and dexamethasone of which functions are stimulation of lipolysis and glycogen synthesis in animals might exert their actions through a leptin-mediated event, and that the role of leptin can be potentiated by these hormones.

The stimulatory effect of insulin on the expression of leptin gene in adipocytes was paralleled up to 75 nM of insulin but no further increase in leptin protein level was observed over 100 nM insulin level in cells, indicating that 100 nM of insulin was sufficient to attain the insulin effect on the expression of leptin gene in adipocytes without any adverse effects. Adipocytes treated with 200 nM of insulin maintained the stimulating effect on leptin gene expression without reduction of cellular viability in this study (data not shown).

Insulin level (100 nM) used in this study was much higher than the level (3 nM) employed by Leroy *et al.* (1996), which was too low to attain a significant effect on leptin mRNA transcription in the present study. But the effect of low level (3 nM) of insulin on leptin gene expression can not be ignored since the application of more sensitive probe for the detection of leptin will make it possible to differentiate the small amount of leptin in cells.

Mouse primary adipocytes prepared in the present study were viable for 28 h of experimental period and well accomodated themselves to the treatment of insulin, epinephrine, growth hormone and dexamethasone (100 nM each), which is the amount used by previous studies (Muller *et al.*, 1997; Yasuda *et al.*, 1997). Since the stimulatory effect of insulin on leptin protein in adipocytes continued during the first 24 h and decreased slightly thereafter, the measurement of leptin in adipocytes and in the culture media was performed at 24 h after the treatment of various hormones.

It has been reported that leptin alleviated insulin effect in rat adipocytes (Muller *et al.*, 1997), implicating that leptin is not only involved in glucose transfer and protein synthesis but also in feed back regulation of fat synthesis. Therefore, it is reasonable to assume that *ob* gene expression in adipocytes may be regulated in response to insulin concentration in cells and that leptin may alleviate the hyperglycemia in diabetes melitus with high insulin resistance (Seufert *et al.*, 1999).

In the present study, administration of insulin to mice increased leptin mRNA transcription in adipose tissue, while growth hormone caused a marginal increase. Consequently, the stimulatory effect of insulin on leptin mRNA transcription was obvious *in vivo* as well as in cell culture system, however growth hormone had little effect on leptin mRNA transcription in mouse adipose tissue.

Growth hormone level in animal tissues is influenced by insulin and obesity, but the regulatory mechanism of body fat decomposition by growth hormone is uncertain (Lanchi *et al.*, 1997; Bercu *et al.*, 1998). Since growth hormone showed little effect on the leptin status in adipocytes in the present study, it is assumed that the decreased endogenous growth hormone secretion in obese tissues observed by previous investigators (Bercu *et al.*, 1998) is thought to be operated in some other mechanisms.

Insulin injection, three times a day for 3 days (0.6 units/mouse), increased leptin mRNA transcription in mouse adipose tissue, but growth hormone injection(0.6 units/mouse) could increase its transcription slightly. Similiar responses to these hormones in cultured adipocytes were also observed in this study. This result confirms the previous report that the expression of leptin mRNA in adipocytes increased transiently 4 h post-injection of insulin to fasted rat and its induction might be influenced by various hormones and factors related to the metabolism of sugars and lipids prior to or after the diet consumption (Saladin *et al.*, 1995).

The facts that insulin administration increased leptin protein level in mouse adipose tissue in the present study support the idea of McDowgald *et al.* (1995) who insisted that insulin plays as a positive regulator for leptin biosynthesis.

Injection of insulin or growth hormone to mice showed no change in leptin level in sera of mice (Table 1), indicating that these hormones may not stimulate leptin secretion *in vivo* during the experimental period and that there might be some different mechanism for the regulaton of blood leptin level.

From these study, it is possible to conclude that insulin or dexamethasone stimulates leptin biosynthesis, which regulates the reduction of food intake and that growth hormone may not stimulate leptin biosynthesis in

mouse adipocytes.

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